From the Department of Neuroscience
Karolinska Institutet, Stockholm, Sweden

BACTERIAL PHYSIOLOGY AND ITS ROLE IN ANTIBIOTIC REFRACTORINESS

Spyridon Gkotzis

Karolinska Institutet

Stockholm 2018

Published by Karolinska Institutet.
Printed by E-Print AB 2018

ISBN 978-91-7676-927-0

Bacterial physiology and its role in antibiotic refractoriness
THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Spyridon Gkotzis

Principal Supervisor:
Assistant Professor Klas Udekwu
Stockholm University
Department of Molecular Biosciences,
The Wenner-Gren Institute

Co-supervisor(s):
Associate Professor Rochellys Diaz Heijtz
Karolinska Institutet
Department of Neuroscience

Associate Professor Andreas Nyström
Karolinska Institutet
Department of Neuroscience

Professor May Griffith
University of Montreal
Maissonneuve-Rosemont Hospital Research Centre and Department of Ophthalmology

Opponent:
Associate Professor Vasili Hauryliuk
Umeå University
Department of Molecular Biology

Examination Board:
Associate Professor Christian Giske
Karolinska Institutet
Department of Laboratory Medicine
Division of Clinical Microbiology

Professor Julia R. Davies
Malmö University
Department of Oral Biology

Professor Jan-Ingmar Flock
Karolinska Institutet
Department of Microbiology, Tumor and Cell Biology

To my North Star(s)

POPULAR SCIENTIFIC SUMMARY

Microbes are living organisms that require a microscope to be seen, with sizes ranging from 0.1 micrometers to a few millimeters. Most of them have a single cell (the smallest unit of life), where a membrane encloses a compartment that contains the basic molecules to sustain life. Humans and microbes have always had a secret partnership, since they are essential in food (e.g. bread) and drink (e.g. wine) production. However, our knowledge of their existence only came in the late 1600s.

The work in this thesis was performed on a subset of microbes called bacteria. Bacteria are everywhere around us; in the air we breathe, in the water we drink, in the food we eat and inside our bodies. In fact, the number of bacteria residing in our bodies is ten times greater than the number of our own cells. Some bacteria are beneficial for us, while others are responsible for a variety of diseases. The drugs used for the treatment of bacterial infections are called antibiotics.

In the first paper, we developed a mathematical model that can explain why bacteria keep dying after removal of the antibiotic, how does the number of bacteria affect a possible treatment with antibiotics and how do persistent bacteria arise. Persistent are those bacteria that do not respond to antibiotic treatment. The model describes the reaction between the antibiotic and the target molecules and thus the resulting effect on bacterial survival. It could be used in the design of optimal antibiotic treatment strategies.

In the second paper we focused on *Staphylococcus aureus*, a bacterium responsible for multiple pathological conditions. Using six different antibiotics currently used in the clinic, we showed that older cultures are harder to kill by antibiotics and tried to provide a rational explanation as to why this happens. Our findings suggest that there is an increasing number of persister cells in aging cultures, directly affecting the number of bacteria surviving treatment.

Some bacteria require oxygen to survive, while some do not. A number of bacteria can survive in both conditions but their characteristics change in the presence or absence of oxygen. In the third paper, we used a *Staphylococcus aureus* that is resistant to one of the most commonly used antibiotics and tried to explain why antibiotic treatment does not have the same effect at different oxygen levels.

The majority of bacteria on the human body live inside our gastrointestinal tract. It is well accepted that our gut bacteria can communicate through chemical signals with our brain. In the last paper we provide evidence of a novel signaling pathway mediating the communication of the gut-residing bacteria and the developing brain.

Overall, bacteria have a profound effect on human health and disease. In this thesis I tried to shed light in some elements that govern the interaction between antibiotics and bacteria. I hope my contribution will spark the interest of future researchers that will expand this knowledge and provide the means for more effective antibiotic treatment.

ΕΠΙΣΤΗΜΟΝΙΚΗ ΠΕΡΙΛΗΨΗ

Οι μικροοργανισμοί είναι μορφές ζωής ορατές μόνο με μικροσκόπιο, με μεγέθη που κυμαίνονται από 0.1 μικρόμετρα έως μερικά χιλιοστά. Οι περισσότεροι από αυτούς έχουν ένα μόνο κύτταρο (η βασική μονάδα της ζωής), όπου μια μεμβράνη περικλείει ένα διαμέρισμα που περιέχει τα βασικά μόρια για τη συντηρήση τη ζωής. Οι άνθρωποι και οι μικροοργανισμοί είχαν πάντα μια μυστική συνεργασία, αφού είναι απαραίτητοι για την παραγωγή τροφίμων (π.χ. ψωμί) και ποτών (π.χ. κρασί). Ωστόσο, η ύπαρξή τους μας έγινε γνωστή μόνο στα τέλη του 16ου αιώνα.

Η διατριβή αυτή πραγματοποιήθηκε σε ένα υποσύνολο μικροοργανισμών που ονομάζονται βακτήρια. Τα βακτήρια είναι παντού γύρω μας: στον αέρα που αναπνέουμε, στο νερό που πίνουμε, στο φαγητό που τρώμε και στο σώμα μας. Στην πραγματικότητα, ο αριθμός των βακτηρίων στο σώμα μας είναι δέκα φορές μεγαλύτερος από τον αριθμό των κυττάρων μας. Ορισμένα βακτήρια είναι ευεργετικά για εμάς, ενώ άλλα είναι υπεύθυνα για μια ποικιλία ασθενειών. Τα φάρμακα που χρησιμοποιούνται για τη θεραπεία βακτηριακών λοιμώξεων ονομάζονται αντιβιοτικά.

Στην πρώτη εργασία αναπτύξαμε ένα μαθηματικό μοντέλο που μπορεί να εξηγήσει γιατί τα βακτήρια συνεχίζουν να πεθαίνουν μετά την απομάκρυνση του αντιβιοτικού, πώς ο αριθμός των βακτηρίων επηρεάζει μια πιθανή θεραπεία και πώς προκύπτουν τα *επίμονα* βακτήρια. *Επίμονα* θεωρούνται τα βακτήρια που δεν ανταποκρίνονται στη θεραπεία με αντιβιοτικά. Το μοντέλο περιγράφει την αντίδραση μεταξύ του αντιβιοτικού και των μορίων στόχων και συνεπώς το αποτέλεσμα που έχει στην επιβίωση των βακτηρίων. Θα μπορούσε να χρησιμοποιηθεί στο σχεδιασμό βέλτιστων στρατηγικών αντιβιοτικής θεραπείας.

Στη δεύτερη εργασία επικεντρώθηκα στον Σταφυλόκοκκο, ένα βακτήριο υπεύθυνο για πολλές παθολογικές καταστάσεις. Χρησιμοποιώντας έξι διαφορετικά αντιβιοτικά που χρησιμοποιούνται σήμερα στην κλινική, δείξαμε ότι οι γηραιότερες καλλιέργειες είναι πιο δύσκολο να σκοτωθούν με αντιβιοτικά και προσπαθήσαμε να δώσουμε μια λογική εξήγηση για αυτό το φαινόμενο. Τα ευρήματά μας υποδεικνύουν ότι υπάρχει ένας αυξανόμενος αριθμός *επίμονων* κυττάρων σε γηραιότερες καλλιέργειες, επηρεάζοντας αρνητικά μια πιθανή θεραπευτική αγωγή με αντιβιοτικά.

Ορισμένα βακτήρια απαιτούν οξυγόνο για να επιβιώσουν, ενώ κάποια άλλα όχι. Μερικά βακτήρια μπορούν να επιβιώσουν και στις δύο συνθήκες αλλά τα χαρακτηριστικά τους αλλάζουν με την παρουσία ή απουσία οξυγόνου. Στην τρίτη εργασία χρησιμοποιήσαμε ένα Σταφυλόκοκκο ανθεκτικό σε ένα από τα πλέον χρησιμοποιούμενα αντιβιοτικά και προσπαθήσαμε να εξηγήσουμε γιατί η θεραπευτική αγωγή με άλλα αντιβιοτικά δεν έχει το ίδιο αποτέλεσμα σε διαφορετικά επίπεδα οξυγόνου.

Η πλειοψηφία των βακτηρίων που ζουν στο ανθρώπινο σώμα βρίσκονται μέσα στο γαστρεντερικό μας σύστημα. Είναι πλέον αποδεδειγμένο ότι τα βακτήρια του εντέρου μπορούν να επικοινωνούν μέσω χημικών σημάτων με τον εγκέφαλό μας. Στην τελευταία

εργασία, παρέχουμε στοιχεία για ένα νέο μονοπάτι σηματοδότησης που μεσολαβεί στην επικοινωνία των βακτηρίων που διαμένουν στο έντερο και του αναπτυσσόμενου εγκεφάλου.

Συνολικά, τα βακτήρια έχουν σημαντική επίδραση στην ανθρώπινη υγεία. Σε αυτή τη διδακτορική διατριβή προσπάθησα να ρίξω φως σε ορισμένα στοιχεία που διέπουν την αλληλεπίδραση μεταξύ αντιβιοτικών και βακτηρίων. Ελπίζω ότι η συμβολή μου θα προκαλέσει το ενδιαφέρον μελλοντικών ερευνητών που θα επεκτείνουν αυτή τη γνώση και θα παράσχουν τα μέσα για πιο αποτελεσματικές θεραπευτικές αγωγές με αντιβιοτικά.

ABSTRACT

The pathological outcome of a bacterial infection depends on the interplay among the host's innate defenses, the virulence arsenal of the pathogen and antibiotic treatment strategies. Understanding this interplay will provide mechanistic insights on antibiotic pharmacodynamics and bacterial pathogenesis, and set the stage for the development of novel therapeutic interventions.

The discovery and subsequent mass production of antibiotics has been one of the greatest achievements in medical history. Regardless of the fact that antibiotics have been used in medicine for more than 70 years, there is no clear mechanistic understanding of their effect on microbial populations in the host, and prediction of antibiotic pharmacodynamics is still complicated. In Paper I, we aimed to develop a simple model that links bacterial population biology and classical reaction kinetics, while rationally explaining complex patterns of antibiotic action (post-antibiotic growth suppression, density-dependent antibiotic effects, and persister cell formation).

The emergence of antibiotic resistance along with the decline in the rate of discovery of new antibiotics has been one the major challenges in modern medicine. Multi-resistant strains (eg. methicillin resistant *Staphylococcus aureus,* MRSA) are responsible for infections with poor resolution and high mortality rates. The majority of the Staphylococci are commensal, however, they can be responsible for a variety of medical conditions caused by infection processes or the direct production of toxins (skin infections, deep tissue infections, toxic shock syndrome, septicemia, endocarditis).

Treatment failure in Staphylococci has been associated with their ability to form biofilms, which have been implicated in chronic and recurring infections. Another physiological state that has been suggested to be of clinical importance is persister cells. Despite the lack of solid evidence on their clinical manifestation, persister cells have also been implicated in chronic infections. In Paper II, we aim to investigate the role of bacterial physiology in antibiotic refractoriness. We provided evidence of biofilm derivation for a significant fraction of persister cells. In Paper III, we investigated the effect of incubation atmosphere on the susceptibility of biofilm-derived cells and demonstrated an increased refractoriness of *S. aureus* biofilm-derived cells under anaerobic conditions.

Polymicrobial communities play a major role in human health and disease. Recent studies have demonstrated that the gut microbiota modulates brain development and behavior. In Paper IV, we aim to investigate peptidoglycan sensing in the developing brain. In this study we provide solid evidence of a signaling pathway mediating the communication between the gut microbiota and the developing brain.

LIST OF SCIENTIFIC PAPERS

I. Abel zur Wiesch P., Abel S., Gkotzis S., Ocampo P., Engelstädter J., Hinkley T., Magnus C., Waldor M.K., Udekwu K., Cohen T.
Classic reaction kinetics can explain complex patterns of antibiotic action. *Science Translational Medicine*, 2015, 7, 287ra73

II. Gkotzis S., Hell E., Udekwu K.
Stationary phase persistence in *Staphylococcus aureus* is driven by biofilm formation. *Submitted manuscript*

III. Gkotzis S., Pastuszek P., Udekwu K.
Anaerobic culture conditions and their effect on the susceptibility of *Staphylococcus aureus* biofilms. *Manuscript*

IV. Arentsen T., Qian Y., Gkotzis S., Femenia T., Wang T., Udekwu K., Forssberg H., Diaz Heijtz R.
The bacterial peptidoglycan-sensing molecule Pglyrp2 modulates brain development and behaviour. *Molecular Psychiatry*, 2017, 22, 257-266

PUBLICATIONS NOT INCLUDED IN THE THESIS

I. Zhang Y., Gkotzis S., Udekwu K.I., Nyström A.M.
Latest highlights in nanoscale imaging, therapy and biophysics. *Nanomedicine*, 2014, 9, 2079-82

II. Alarcon E.I., Udekwu K.I., Noel C.W., Gagnon L.B., Taylor P.K., Vulesevic B., Simpson M.J., Gkotzis S., Islam M.M., Lee C.J., Richter-Dahlfors A., Mah T.F., Suuronen E.J., Scaiano J.C., Griffith M.
Safety and efficacy of composite collagen-silver nanoparticle hydrogels as tissue engineering scaffolds. *Nanoscale*, 2015, 7, 18789-98

III. Griffith M., Udekwu K.I., Gkotzis S., Mah T.F., Alarcon E.I.
Anti-microbiological and anti-infective activities of silver. In: Alarcon E., Griffith M., Udekwu K. (eds) Silver Nanoparticle Applications. Engineering Materials. Springer, Cham. 2015

CONTENTS

LIST OF ABBREVIATIONS

CCCP	Carbonyl cyanide m-chlorophenyl hydrazone
CFU	Colony forming unit
E. coli	*Escherichia coli*
eDNA	Extracellular DNA
EPS	Extracellular polymeric substances
FMT	Fecal microbiota transplantation
MIC	Minimum inhibitory concentration
MRSA	Methicillin resistant *Staphylococcus aureus*
PCR	Polymerase chain reaction
PIA	Polysaccharide intercellular adhesin
qRT-PCR	Quantitative real time PCR
S. epidermidis	*Staphylococcus epidermidis*
S. aureus	*Staphylococcus aureus*
SCV	Small colony variants
UTI	Urinary tract infection
WT	Wild type
WHO	World Health Organization
σ^B	Alternative sigma factor B

1 BACKGROUND

1.1 ANTIMICROBIALS

Antimicrobials are chemical substances that inhibit or kill microorganisms. They can be derived from other microorganisms or synthetically produced. Special emphasis in this thesis will be given to antibiotics. The term antibiotic is given to any drug that can be used to treat bacterial infections. Antibiotics are usually low molecular weight organic compounds, produced as secondary metabolites by microorganisms that selectively inhibit the growth of other microorganisms. An overview of the different classes of antibiotics and their targets is summarized in Figure 1. Evolutionarily, the role of these molecules has been hypothesized to be to confer an ecological advantage for survival in natural environments where resources are limiting for bacterial growth.

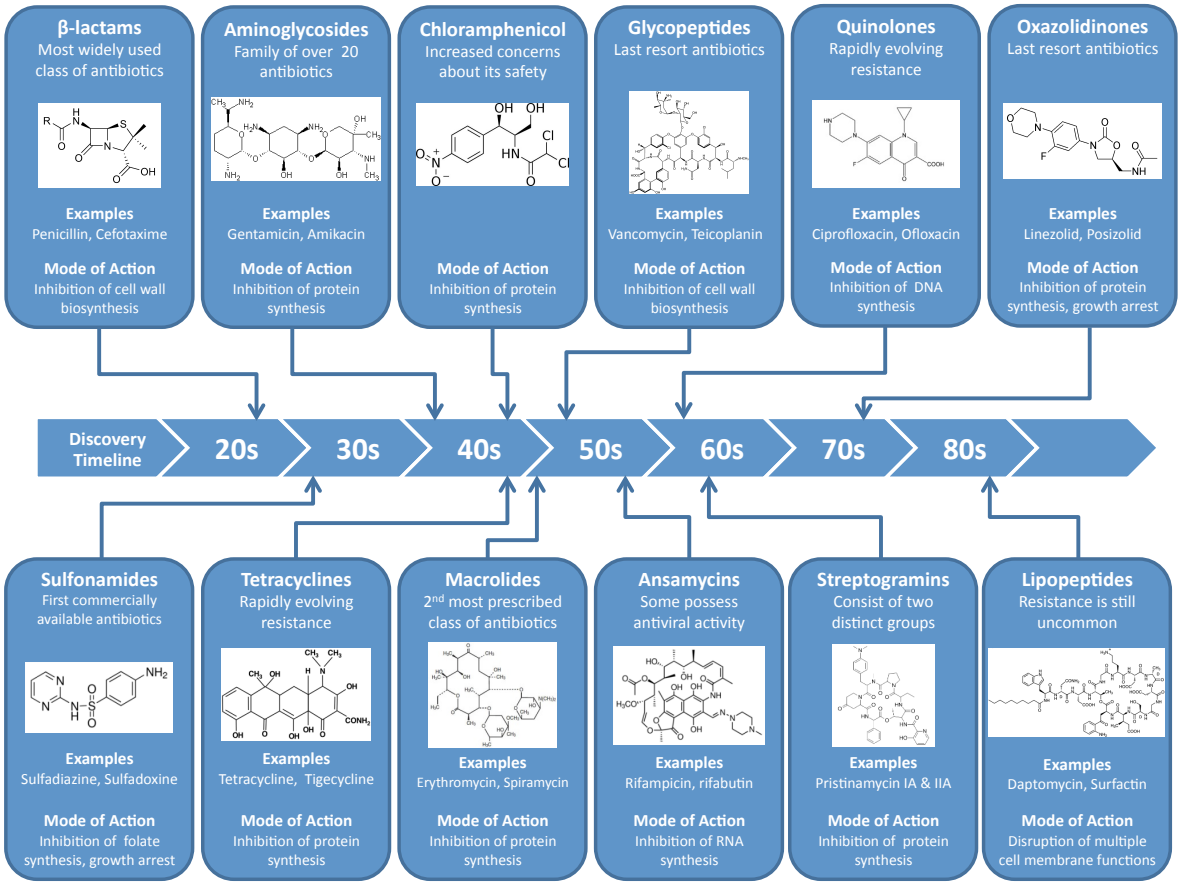

Figure 1. An overview of the different classes of antibiotics

Long before the introduction of antibiotics, different civilizations in the course of history had utilized beer yeast, mold and mushrooms to treat infected wounds. In ancient Egypt and Greece, physicians used compresses and tonics made from herbs, molds and organic compounds to treat patients. Despite the fact that they were unable to explain the reason of

this effect, they harnessed the healing powers of natural compounds. Empirical treatments dominated medicine up until the discovery and description of bacteria.

The discovery and subsequent mass production of antibiotics in the beginning of the 20th century was one of the most important achievements in modern medicine. The introduction of antibiotics, along with improvements in hygiene measures and a comprehensive understanding of pathogenic microorganisms, was instrumental for restricting many infectious diseases and had a profound positive effect on life quality and life expectancy.

The first natural antibiotic to be described in modern history was discovered by a young medical scientist, Bartolomeo Gosio [1]. He purified a substance from fungi growing on corn that exhibited activity against *Bacillus anthracis* (the causative agent of anthrax). This compound was some years later identified as mycophenolic acid. Ehrlich and Bertheim discovered the first synthetic antibiotic in 1909, which was an arsenic derivative called arsphenamine. Within less than a year from its discovery, arsphenamine (marketed as Salvarsan) was used for the clinical treatment of syphilis by 1910 [2].

Sulfonamides were the first class of antibiotics to be introduced in the clinic in the early 1930s [3]. Prontosil was marketed in 1935 and was very effective against gram-positive cocci (mainly *Streptococcus*) drastically reducing deaths from pneumonia, meningitis and childbed fever. However, it was the work of Sir Alexander Flemming that revolutionized antimicrobial treatment. Even though penicillin was first discovered (accidentally) in 1928, it was not until the early 1940s that it started being mass-produced and used in patient therapy. The discovery of beta-lactam (β-lactam) antibiotics is a hallmark in modern chemotherapy. The second half of the 20th century is dominated by the discovery of many new and important antibiotics, both natural and synthetic, with a wide range of activity.

Antibiotics have long been indispensable for infection treatment but their indiscriminate use has driven the ascent and dissemination of antibiotic resistance. Many antibiotics are losing - or have already lost- their potency against major pathogens. This antibiotic resistance is brought about via five basic paths:

- Modification of the antibiotic target molecule with mutation being the key factor.
- Destruction of the antibiotic before entering the cell via cleavage by specialized enzymes.
- Modification of the antibiotic molecule that leads to inactivation.
- Active pumping of the antibiotic outside the cell with the use of specific or non-specific transport proteins.
- Reduced uptake of the antibiotic by bacterial cells.

Upon discovery, antibiotics were considered the “magic bullet” against all pathogenic microorganisms, leading to the US surgeon General in 1968 to declare that the battle against infections was won. It soon became clear that this notion was far from the truth. Bacteria utilize a very diverse arsenal while evading the host’s defense mechanisms in order to establish themselves in host organisms. Through drug evasion and/or degradation, primary

resistance, phenotypic resistance, sub-optimal growth and residence in protected environments (intracellularly or in biofilms), bacteria manage to persist during antibiotic exposure.

A very good example showing the potential clinical relevance of these mechanisms is that of community-acquired pneumonia patients infected by susceptible strains of *Streptococcus pneumoniae* that are unresponsive to antibiotic therapy [4]. As a consequence of the above-stated mechanisms of survival in lieu of primary resistance, the question why antibiotic therapy fails arises. A valid answer is the evolution of resistance and the dissemination of resistance cassettes horizontally among non-related species. However, these two mechanisms cannot account for all clinical failure. Antibiotics can sometimes fail due to a non-inherited refractoriness that results from differential physiology. This physiology is in turn affected by population age, physical structure, antibiotic pre-exposure and quorum sensing amongst others.

The rate of discovery for new antibiotics or antimicrobial agents has been in decline over the last three decades, and taken together with the spread of drug multiresistance, the need for alternative methods to combat infection is evident. Now, more than ever, is imperative to experimentally identify optimal regimens that will both conclude in successful patient treatment and also suppress the emergence of resistance.

1.1.1 Antibiotics used in this study

Ampicillin

Ampicillin is a β-lactam antibiotic of the penicillin class with broad-spectrum activity and listed in the World Health Organization's (WHO) "List of essential medicines" [5]. It is an irreversible inhibitor of the enzyme transpeptidase, which is essential in bacterial cell wall biosynthesis. It has traditionally been used for the treatment of respiratory tract infections, urinary tract infections (UTIs), meningitis, and endocarditis.

Ciprofloxacin

Ciprofloxacin is a second-generation fluoroquinolone with broad-spectrum activity, also listed in the WHO's "List of essential medicines" [5] . It inhibits the function of DNA gyrase, and a type II topoisomerase (topoisomerase IV), both necessary in bacterial DNA replication [6]. It has been used for the treatment of a wide range of conditions, such as skin and deep tissue infections, typhoid fever, respiratory tract infections, UTIs, and sexually transmitted diseases.

Daptomycin

Daptomycin is a lipopeptide antibiotic mostly used in infections caused by gram-positive bacteria. It has a distinct mechanism of action, inhibiting a number of different cell

membrane functions. More specifically it inserts into the cell membrane and aggregates. This aggregation alters the curvature of the membrane inducing the formation of holes that leak ions. The depolarization and resulting loss of membrane potential leads to rapid bacterial cell death [7]. Daptomycin has been used to treat infections caused by multiple drug-resistant bacteria. Most commonly it has been used against *Staphylococcus aureus* (*S. aureus*) bacteraemia and endocarditis, and other gram-positive mediated skin infections.

Gentamicin

Gentamicin belongs to the aminoglycoside class and is listed in the WHO's "List of essential medicines" [5]. It is mostly active against gram-negative bacteria, but also the gram-positive Staphylococci. It irreversibly binds the 30S subunit of the bacterial ribosome, thus interrupting protein synthesis. It has a wide range of applications and has been used against pneumonia, meningitis, UTIs, endocarditis, sepsis and deep tissue infections.

Linezolid

Linezolid is a member of the oxazolidinone class and is also part of the WHO's "List of essential medicines" [5]. It is used for the treatment of infections caused by almost all clinically important gram-positive bacteria that are resistant to other antibiotics. It binds to the 23S rRNA of the 50S ribosomal subunit occupying the A site. There, it induces a conformational change that prevents tRNA from entering the site and forcing tRNA to fall off the ribosome, ultimately blocking initiation of translation [8]. Linezolid is considered bacteriostatic against most organisms *in vitro*, however *in vivo* bactericidal action can be exhibited by inhibition of toxin production in Staphylococci and Streptococci. It is used in the treatment of skin infections, pneumonia and drug-resistant tuberculosis.

Nalidixic acid

Nalidixic acid is a first-generation fluoroquinolone mostly effective against gram-negative bacteria. It has the same mechanism of action as ciprofloxacin. Historically it had been used for the treatment of UTIs, however, now it has been replaced in the clinic with more effective and less toxic drugs.

Oxacillin

Oxacillin is a β-lactam antibiotic of the penicillin class with a narrow spectrum of activity. It inhibits the synthesis of the peptidoglycan layer of bacterial cell walls by binding to the active center of penicillin-binding proteins and preventing the final cross-linking of the growing peptidoglycan layer. It is penicillinase-resistant and consequently has been used widely to treat penicillin-resistant *S. aureus* related infections.

Streptomycin

Streptomycin is an aminoglycoside with broad spectrum activity and included in the WHO's "List of essential medicines" [5]. It binds to the 16S rRNA of the 30S subunit of the bacterial

ribosome, interfering with the binding of the formyl-methionyl-tRNA, which in turn induces frame-shift mutations and defective protein synthesis [9]. It is used to treat endocarditis, tuberculosis (in combination with other antibiotics), brucellosis and plague, among other conditions.

Tetracycline

Tetracycline belongs to the tetracycline class of antibiotics and also listed in the WHO's "List of essential medicines" [5]. It has a broad range spectrum of activity and has been used for the treatment of a variety of conditions such as acne, cholera, brucellosis, plague, malaria and syphilis. It binds to the 30S subunit of bacterial ribosomes where it blocks the attachment of charged aminoacyl-tRNA to the A site, thus inhibiting protein synthesis in susceptible bacteria.

Vancomycin

Vancomycin is a type of glycopeptide antibiotic and indexed in the WHO's "List of essential medicines" [5]. It recognizes and binds to the two D-alanine residues on the end of the peptide chains preventing cross-linking of the growing peptidoglycan layer. It is considered a last resort medication for the treatment of septicemia and lower respiratory tract, skin, and bone infections caused by gram-positive bacteria.

1.1.2 Antimicrobial susceptibility testing methods

Antimicrobial susceptibility testing may be the single most important activity performed in the clinical microbiology laboratory [10]. It is of paramount importance to reliably detect antibiotic resistance in microorganisms and ensure a successful antimicrobial treatment. A number of different methods have been developed and each of them has its own advantages and disadvantages, as well as to which microorganisms can be accurately used on. Some of the methods provide quantative results (minimum inhibitory concentration) while others offer qualitative results (susceptible, intermediate or resistant). Nevertheless, all of the methods are either based on confirming susceptibility or detecting resistance to antimicrobial agents.

Dilution methods

The broth dilution method is based on challenging a microorganism with a series of concentrations of antimicrobial agents in broth environment. There are two variations of this method depending on the broth volume; microdilution testing in volumes of 100 µl and macrodilution testing in volumes up to 1 ml. The latter was one of the first antimicrobial susceptibility methods to be developed [11]. For both methods, a two-fold dilution of an antibiotic is prepared in a liquid growth medium. A standardized bacterial inoculum of 1–5 x 10^5 colony forming units per ml (CFU/ml) is then added, allowed overnight incubation at 35 ^oC and then turbidity is assessed [12]. The lowest concentration of an antibiotic that visibly

inhibits the growth of the microorganism is recorded as the minimum inhibitory concentration (MIC).

A similar technique has been developed for MIC assessment on agar plates called agar dilution method. This method follows the same principle to identify the lowest concentration of the serially diluted antibiotic at which bacterial growth is still inhibited.

The major advantages of these techniques have been the quantitative output, presented by a defined MIC concentration, and reproducibility. The precision of this technique is plus or minus a dilution step. Among the major weaknesses of the dilution methods are the long time to prepare the experiment, possible pipetting errors and errors in preparation of the antibiotic solutions.

Disk diffusion method

The disk diffusion susceptibility method is very simple and has been well standardized [13]. An inoculum of approximately 1-2 x 10^8 CFU/ml from the microorganism is evenly seeded on an agar plate. Commercially prepared disks, each of which is pre-impregnated with a standard concentration of a particular antibiotic, are then dispensed onto the agar surface. The antibiotic begins to diffuse outward from each disk, creating a gradient of antibiotic concentration in the agar. The plates are allowed to incubate for 16–24 h at 35 ^oC and the bacterial growth around the disks is monitored. If the microorganism is susceptible, a clear zone of inhibition will be observed around that disk. The diameter of the zone is measured in millimeters and then compared to an interpretation chart [12] allowing classification of the microorganism as susceptible, intermediately susceptible or resistant. No actual MIC value can be obtained with this test.

This method is gaining popularity due to its convenience, efficiency and cost, and it is probably the most widely used method for determining antimicrobial resistance patterns in clinics. Most likely the major disadvantage of this method is the lack of automation. Moreover, according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) a number of organism-antimicrobial agent combinations do not provide reliable results [14].

E-test

E-test (AB Biodisk, Sweden) is a commercially available test that utilizes a plastic strip impregnated with a gradually decreasing concentration of an antibiotic on the bottom side, while the upper surface has a concentration scale. A suitable agar plate is inoculated with a standardized inoculum as in the disk diffusion method mentioned above. Up to six different strips can be arranged in a radial fashion on the agar surface. Plates are then incubated overnight and the MIC is determined by the intersection of the lower part of the ellipse shaped growth inhibition area with the test strip.

This method is a convenient quantitative test of antibiotic resistance that has been used to assess clinical isolates. However, a separate strip is needed for each antibiotic, and therefore the cost of this method can be quite high. In general, results obtained with E-test have correlated well with MICs generated by broth or agar dilution methods [15]. Conversely, certain organism-antimicrobial agent combinations are known to show systematic biases when assessed by E-tests [16].

Automated antimicrobial susceptibility testing methods

A number of different commercial systems have been developed to facilitate high throughput of antimicrobial testing [17]. The actual assessment of antimicrobial susceptibility is based on broth microdilution method, with these automated systems providing inoculation, reading and interpretation. Despite the high sample throughput and convenience, such systems can be quite expensive to purchase and maintain in laboratory settings. Some examples of these include: Vitek 2 System (bioMerieux), Sensititre ARIS 2X (Trek Diagnostic Systems), MicroScan WalkAway (Siemens Healthcare Diagnostics) and Phoenix (BD Diagnostics).

Mechanism-specific tests

Such tests are based on directly detecting particular resistance mechanism(s). They are usually developed for antibiotics with a well-understood background on resistance acquisition. Some examples are the chromogenic cephalosporinase test [18] used to detect the presence of β-lactamases and the chloramphenicol acetyltransferase (CAT) reagent kit [19] that detects the presence of this chloramphenicol-modifying enzyme.

Genotyping methods

When resistance to a given antibiotic is genetically encoded, there are methods to evaluate the presence of specific genes that confer antibiotic resistance. Two of the most common molecular techniques used in antimicrobial resistance detection are: Polymerase Chain Reaction (PCR) and DNA hybridization.

PCR based methods are the most commonly employed. They are based on the direct amplification of known resistance genes and can provide highly reliable and rapid results. DNA hybridization techniques are based on direct identification of target sequences with specifically designed probes. When hybridization occurs, a signal (enzymatic, radioactive or luminescent) is produced; if the sequence in question is not present no signal will be detected. Both approaches offer high sensitivity and specificity in detection.

Even though nucleic acid-based systems are rather fast and inexpensive, the presence of a resistance-related gene does not necessarily result in treatment failure because resistance is also dependent on the mode and level of expression of these genes. Moreover, these techniques have limited utility, since only a few resistance genes are firmly associated with phenotypic resistance.

1.2 BIOFILMS

The concept of bacteria living in sessile communities, knowledge we now take for granted, has not been so widespread in the past. The first publication to acknowledge bacterial growth on surfaces comes from the works of the zoologist Claude Zobell in 1943 [20]. Within the forty following years there were sporadic reports investigating surface-associated bacterial growth and their role in natural environments and in pathology. It was only in 1987 when surface-associated lifestyle was termed biofilm and was recognized as the major form of existence for many microorganisms [21].

A biofilm is defined as "a structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface" [22]. Biofilms are universal [23,24] and all surfaces that come in contact with a naturally occurring liquid are susceptible to this form of bacterial colonization. Mature biofilms are characterized by intricate three-dimensional structures that have channels and pores allowing exchange of material with the environment [24]. Their architecture varies and can be influenced by a variety of factors (e.g. internal pH, carbon source, oxygen perfusion, location, nature of comprising bacteria and osmolarity, among others) [25,26].

It is generally accepted that biofilms constitute a protected mode of life when environmental conditions are not favorable. It has also been suggested that biofilm-residing bacteria metabolically perform better than their planktonic counterparts [27]. Biofilms function as gene pools and greatly facilitate exchange of genetic material mostly by horizontal gene transfer [28]. The biofilm mode of life is very successful among bacteria, but also extends to other life domains like archea [29], fungi [30] and algae [31]. In most natural environments biofilms are inhabited by a large number of different species, where the first colonizer usually conditions a surface and provides a holdfast for the attachment of other colonizers, and/or the metabolic by-products of one might serve as nutrients for the other [27,32].

It is now evident that in medical, industrial and natural settings the formation of biofilm seems to be the most common mode of life. As a strategy it demonstrates a selective advantage, coupled with a higher probability of survival for those sessile bacteria [21,22]. Biofilms exhibit a remarkable ability to resist a variety of biocides and this resistance can be up to 1000-fold higher than that of planktonic cultures in some cases [33]. Moreover, biofilms have been associated with increased resistance against host defenses and immuno-evasion [34-36]. This tenacity of biofilms, combined with the high refractoriness of biofilm-associated infections, calls out for new approaches in combating such infections.

1.2.1 BIOFILM MATRIX

Biofilms have two major components: cells and the matrix enclosing them (Figure 2). The matrix holds the biofilm together and accounts for many of its functions [37]. In addition to acting as a physical barrier protecting the cells, it facilitates cell-to-cell communication

through specialized biochemical signals [38]. The matrix is composed of materials called extracellular polymeric substances (EPS), mainly comprised of complex polysaccharides, proteins, extracellular DNA (eDNA), amyloid fibers and lipids [37]. The major components though, are carbohydrates and proteins [39].

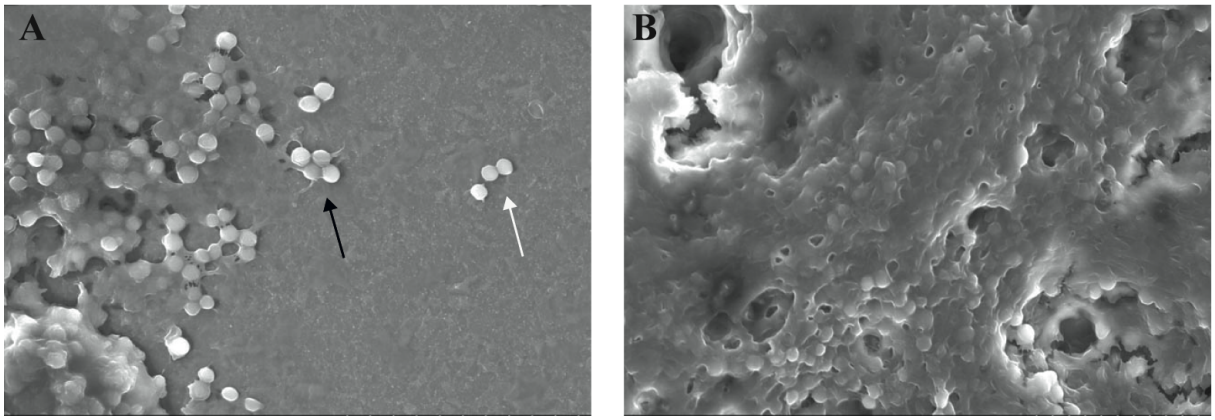

Figure 2. Scanning electron micrographies showing biofilm formation. After the initial attachment on a surface (white arrow in A) the bacteria start producing EPS components (black arrow in A) and eventually they are encased by the biofilm matrix (B) (Modified from [40]).

Matrix composition has a great impact on a biofilms' physicochemical properties and subsequently on its survival. It affects nutrient adsorption, mechanical stability, density, porosity and hydrophobicity, among others that are vital for biofilm survival [41]. It protects the enclosed cells from environmental stress factors (e.g. dehydration, predation, oxidation stress) and treatment from antimicrobial agents. Despite the fact that the exact content of the matrix differs from one biofilm to another, there is speculation of some characteristics being shared by all of them [37].

Microbial activity in biofilms is defined by the composition, structure and properties of the polymers that comprise the matrix [42]. These polymers have effects on oxygen diffusion, substrate absorption and transport of molecules throughout the matrix. The chemical composition and physical properties of the polysaccharides vary significantly among biofilm matrices. The differences can be on the type of monomers that constitute the polysaccharides, the type of glycosidic linkages (β-1,4, β-1,3 or α-1,6) and the frequency of different organic and inorganic substitutions. Characterization of EPS components is essential in understanding the structure-function relationship in biofilms.

Another important function of the matrix is to facilitate cell-to-cell communication. Within the biofilm structure -where high bacterial densities are achieved- small molecules are excreted facilitating communication and thus allowing the bacteria to modify their behavior in a coordinated manner [38]. This process has been called quorum sensing and it is strongly related to virulence and biofilm dispersal mechanisms. Moreover, such close proximity of

cells allows for greater chances of successful genetic material exchange when compared to planktonic cultures [43]. Lastly, the matrix acts as a barrier retaining a pool of extracellular enzymes in the cells' vicinity and limits diffusion of nutrients from lysed cells.

1.2.2 BIOFILM DEVELOPMENT

Biofilm formation is a complex process that involves a coordinated gene expression and a tight regulation of a big set of diverse genes. The development of a biofilm can be summarized in 5 steps: i) reversible attachment, ii) irreversible attachment, iii) maturation I, iv) maturation II and v) dispersal [44] (Figure 3).

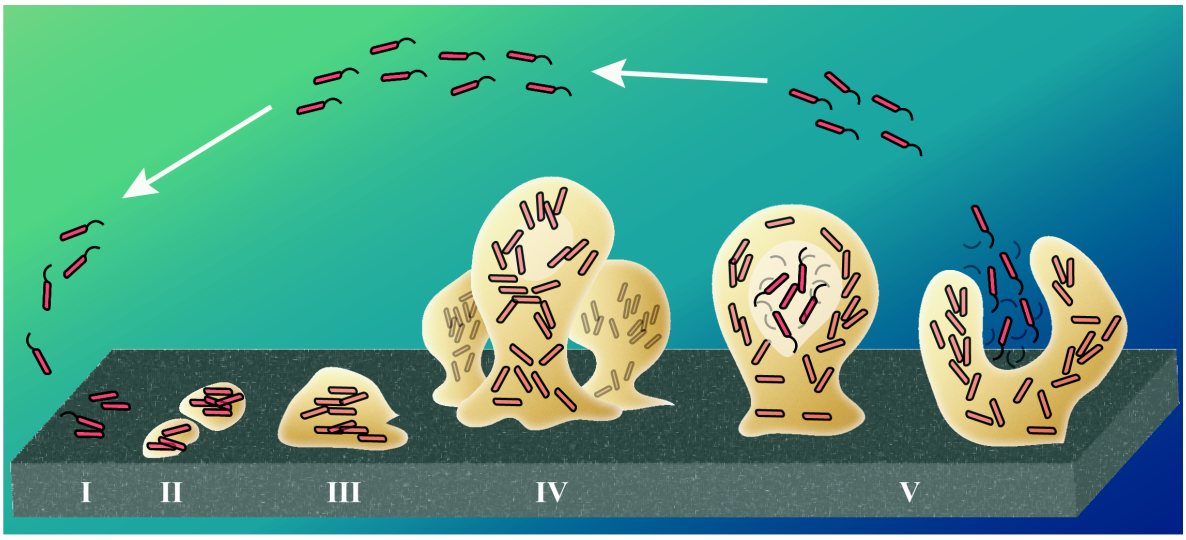

Figure 3. The five developmental stages of biofilm formation. I) reversible attachment, II) irreversible attachment, III) maturation I, IV) maturation II and V) dispersal

Reversible attachment

The first step in establishing a biofilm is the close proximity of a bacterium and a surface. Bacteria can reach a surface actively by chemotaxis and motility or by chance through liquid flow. Whether attachment is favored or not depends on the net repulsive forces (electrostatic interaction, Van der Waals, hydrodynamic forces, hydrophobic interaction) occurring when the distance between cell and surface is approximately 1 nm. Different approaches are employed by bacteria to attach to different materials/surfaces. In general, attachment to abiotic surfaces is mainly non-specific, while attachment to biotic surfaces utilizes specialized molecular docking appendices (e.g. pili, adhesins, fimbriae) [45].

Irreversible attachment

This initial step is a dynamic process and cells can detach from the surface since attachment is reversible. Once attachment is favored, it becomes non-reversible with the production of EPS material and/or the induced expression of adhesive proteins. By the end of this phase, bacterial cells are steadily fixed on the surface.

Maturation I

The next phase involves additional production of adhesion molecules and cell division. Biofilm matrix is being produced and the attached cells give rise to microcolonies. This phase concludes with the early architectural characteristics of the biofilm being present.

Maturation II

During this phase biofilm complexity achieves its maximum capacity. The bacteria reach a maximum density and continuously produce extracellular polymeric substances giving rise to intricate three-dimensional structures. The mature biofilm extends towards its environment and is dominated by channels and pores that allow liquid flow through the structure [24]. Biofilm architecture is not consistent, and factors such as internal pH, availability of carbon sources, oxygen perfusion, location, nature of comprising bacteria and osmolarity have been shown to affect it [25,26].

Dispersal

The last stage in the biofilm mode of life is dispersal, when bacteria leave the biofilm and return to a planktonic lifestyle. It can occur in two ways: naturally or by external forces [46]. The natural dispersal is a highly regulated process that depending on the environmental conditions allows for extended colonization of a favorable niche or triggers the bacteria to search for new locations [47]. On the other hand, biofilm colonization can occur by external forces such as physical detachment or shear forces.

1.2.3 SIGNIFICANCE OF BIOFILMS

Biofilm related infections account for approximately 65% of the hospital-acquired infections [48]. Their ability to form on a variety of surfaces, and more specifically within indwelling medical devices (e.g. catheters, prosthetic devices), draws attention to their vast clinical impact. There is compelling evidence that biofilms contribute to chronic as well as latent infections [49,50]. They have been also correlated with high risk of medical implant rejection with associated severe complications [51]. Oral biofilms, on the other hand, have been linked with a variety of pathological conditions [52].

Biofilms do not only have clinical implications but are also involved in health risks in daily life. Examples of this are found in the food and beverage industry. Biofilms forming on food

products are generally hard to eradicate, thus compromising food quality [53]. Moreover, biofilm emerging inside water pipes can have a negative effect on water quality.

In industrial settings biofilms can lead to great financial damage. A well documented example is the oil industry, where biofilm forming in distribution pipes can lead to corrosion, blockages or even spoil the oil or gas [54]. Another common issue is biofouling, where biofilm-related communities cause corrosive damage or potential blockages.

Despite their negative impact in clinical and industrial settings, biofilm formation can be harnessed for beneficial purposes. Biofilm communities have been used as molecular filters in sewage treatment and for bioremediation in contaminated marine systems [55].

1.3 PERSISTERS

Shortly after the introduction of penicillin, a physician named Joseph Bigger observed that increasing concentrations of penicillin added to a culture of *S. aureus* induced lysis, but the lysed material gave rise to fresh colonies upon plating. Bigger concluded that penicillin was not able to sterilize an infection and named the surviving cells "persisters" [56]. It has been 70 years since the observation of persister cells and we still do not have a complete answer regarding the mechanism of their formation.

"Bacterial persister cells are dormant variants of regular cells that form stochastically in microbial populations and are highly tolerant to antibiotics" [57]. Persistence is to be differentiated from antibiotic resistance since persister cells do not differ genetically from their susceptible counterparts in a population. Indeed, cultures grown from persisters are as sensitive to the drug as the parental culture the persisters originated from [58]. It is generally accepted that persistence arises from phenotypic population heterogeneity within an isogenic population. Balaban *et al.* have proposed that there are two types of persister cells: type I cells that exhibit a reduced exit rate from stationary phase, as opposed to type II cells that constantly interconvert between the persister and susceptible state [59].

The tolerance of persister cells has mainly been associated with entry to a non-growing physiological state where the cell metabolism is kept to the minimal essential required for survival. The physiology of persisters is often compared with that of stationary phase cells. Indeed, stationary phase cultures have been extensively used as a model of non-growing population and most of the understanding we have about persisters comes from challenging these cultures with different antibiotics [60,61]

Experimental study of persisters is challenging due to a number of complications. Most notably, current data suggest that the persister population is not stable with a constant interconversion between the persister and susceptible state [59,62]. The number of persisters is really small in a given culture, with only 1 in 10^5-10^6 cells in a wild type *Escherichia coli* (*E.coli*) culture [63]. Moreover, when identical cultures are exposed to different antibiotics there

is substantial difference in persister levels [61,64]. In order to begin understanding what persisters are, one must keep in mind that they are a heterogeneous population with physiological diversity arising from cell to cell variability in any number of cellular processes (Figure 4).

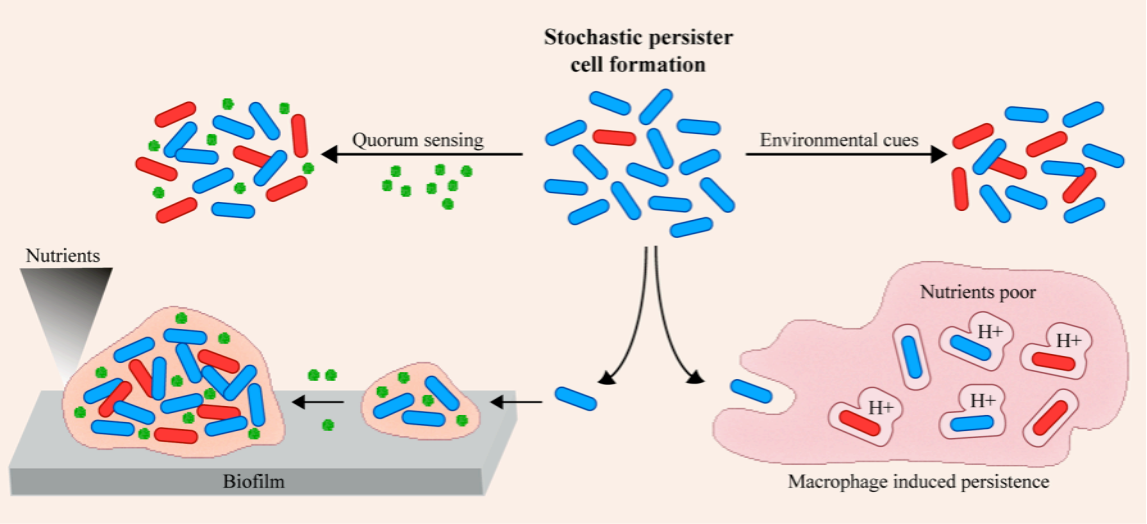

Figure 4. Persister cell formation can be stimulated by environmental and physiological cues.

The first evidence of a genetic link to persister formation has been the discovery of the *hipA7* gain of function allele [58]. However, a deletion of the *hipA* gene showed no relative phenotype. In recent years, the increased availability of different knockout and transposon mutant libraries has led to a more straightforward approach based on screening towards the molecular characterization of persistence mechanism. A screening of the *E. coli* gene knockout KEIO collection [65] failed to identify any gene that eliminated persister formation [60]. In the same study, only a number of global regulators knockouts showed a 10-fold decrease in persister formation, suggesting a higher degree of redundancy in whatever mechanism(s) might be involved in persister formation. Screening studies in other species produced similar results [66].

A number of genetic studies have linked persistence to toxin-antitoxin systems [67], stringent response [68], levels of DNA damage [69] and more recently changes in metabolic pathways [70]. These observations strengthen the argument of redundant pathways and functional overlap in persister formation. It is evident that the cellular metabolic state seems to be an important cue driving persister formation. Moreover, the role of DNA damage repair systems [69] adds towards the argument that persister formation might be inducible [59].

Bacterial persistence has often been perceived as a model of survival in fluctuating environments. As elegantly stated by Kusell *et al.*, "persisters are an insurance policy against antibiotic encounters" [71]. It is an epigenetic strategy to reduce the risk associated with fast

growth in nutrient rich environments. A fraction of the population is characterized by suppressed growth when the remaining population grows exponentially. The lowering of the population fitness is in fact a risk-reducing solution that improves the odds of survival of a population under any type of stress (antimicrobials, host defenses or environmental factors).

Some promising studies have revealed enhanced clearance of persisters from infection models or reduced persister formation in *in vitro* systems. Li & Zhang found that inactivation of *phoU* resulted in enhanced bacterial death for many antibiotics and environmental stresses [61]. Notably, a study from Allison *et al.* established a strategy to remove persisters in a mouse UTI model through aminoglycoside potentiation by specific metabolic stimuli [72]. More recently, Lebeaux *et al.* demonstrated the ability of pH-mediated potentiation of aminoglycosides to eradicate persisters in catheter-related infections [73].

Persister cells have been suggested to play a major role in recurring chronic infections [74,75]. High persister mutants have been isolated from a number of infections regardless of site of infection and species, such as *Pseudomonas aeruginosa* from cystic fibrosis patients [76] or *Candida albicans* from oral thrush biofilm [77]. Furthermore, they have been implicated in tolerance not only to antibiotics but also to other toxic molecules such as heavy metals [78]. The failure of most antibiotics to control persister populations only highlights the importance of understanding the molecular mechanism(s) governing persister formation.

1.4 SMALL COLONY VARIANTS (SCV)

Small colony variants were first described in 1935 for *S. aureus* [79] and soon after in various coagulase-negative Staphylococci. At first they were believed to be part of the normal bacterial cell cycle, but it took almost 80 years to show that phenotypic switching between normal and SCV phenotypes occurs during exponential growth even in the absence of any selective pressure [80]. The SCV phenotype is linked to chronic, recurrent, and antibiotic-resistant infections [81]. Occurrence of SCVs is not limited to human clinical infections, but is also found in veterinary infections [82] and in the food industry [83].

The molecular mechanism behind SCV emergence has not yet been fully elucidated. Until now six different pathways/mechanisms have been well described: menadione-dependent [84], hemin-dependent [85], thymidine-dependent [86], fusidic acid-resistant [87], with an impaired stress response [88] and with impaired stress response to cold shock [89]. Ongoing research supports the notion that some SCVs might rise as an effect of yet unrecognized regulatory genes, due to the fact that a portion of them return to normal phenotype upon subculture [90].

SCVs have been associated with invasion of mammalian cells and intracellular persistence [91,92]. Genetic and expression studies of SCVs have provided numerous clues to support the above notion. *S. aureus* SCVs have been described with high expression of adhesins and production of fewer lytic enzymes that facilitate persistence and host cell uptake [91].

Moreover, SCV phenotypes are highly associated with down-regulation of the tricarboxylic acid cycle and other metabolic pathways [93].

Along with the formation of biofilm and persister cells, SCVs have been suggested to be a bet hedging strategy under stress conditions in the form of a phenotypic switch [94]. It is generally accepted that the emergence of SCVs is enhanced in strains with mutator phenotypes, which are affected in their DNA repair systems [85]. SCVs are then selected either by environmental stress conditions [83] or by intracellular location [95]. Due to the inherent difficulties in successfully treating infections associated with SCVs, more studies to optimize antibiotic therapy are needed.

1.5 STAPHYLOCCOCUS AUREUS

1.5.1 INTRODUCTION

Staphylococcus aureus is a gram-positive coccus classified in the Firmicutes phylum. It is a facultative anaerobe that grows in clusters, often in hexagonal arrangement. Studies have shown that 1 out of 3 people are asymptomatic carriers, with the bacterium being part of their normal microbiota. It mostly resides on the skin, the respiratory tract and the perineum [96,97].

The genome has approximately 2.8 million base pairs and may include one or more plasmids. Currently, there are 162 complete genome sequences available for *S. aureus* strains in the National Center for Biotechnology Information (NCBI) genome database and almost 8000 genome assemblies have already been deposited [98]. The general consensus is that approximately 75% of the genome is conserved among sequenced strains, but with each new strain being sequenced it is evident that the variable element is only growing.

Although *S. aureus* is not always pathogenic, it is a common cause of a variety of conditions with diverse severity. Staphylococcal infections include minor skin infections, boils, wound infections, toxic shock syndrome, pneumonia, osteomyelitis, endocarditis, bacteraemia and sepsis, among others (see [99] for a more comprehensive review). The primary site of infection is the skin or superficial wounds [100], from where the organism can gain access to the bloodstream and invade host tissues with a variety of clinical manifestations. It is estimated that approximately half a million patients are hospitalized due to staphylococcal related infections in the USA alone [101].

People with underlying chronic conditions such as diabetes, cancer, vascular disease, eczema, and lung disease are at higher risk of developing staphylococcal infections. Moreover, hospitalized patients are also at high risk, especially when immuno-compromised. *S. aureus* is the most commonly isolated bacterium in chronic wounds (93.5 %) followed by *Enterococcus faecalis* (71.1 %), *Pseudomonas aeruginosa* (52.2 %) and coagulase-negative Staphylococci (45.7 %) [102]. Equally important, *S. aureus* and *S. epidermidis* are responsible for the majority of biofilm-mediated device-related infections in healthcare settings [103].

S. aureus is a highly adaptable microorganism with a broad niche and host range, that has often been associated with chronic and/or persistent infections [22,49]. In general, staphylococcal virulence has been linked with the ability of the organism to grow in biofilms [104]. However, biofilm formation in *S. aureus* will be discussed in more detail in the following section. Another disconcerting observation is the high rate of resistance acquisition to last resort antibiotics, such as vancomycin [105]. The rise of multi-resistant strains is an ongoing problem for healthcare settings resulting in increased morbidity and mortality worldwide. The adeptness of *S. aureus* to survive and adapt to a spectrum of different environments only stresses the complications involved in its eradication.

The rise of MRSA coincides with the conclusion of one year from the clinical introduction of methicillin for the treatment of staphylococcal infections [106]. Since then, MRSA has been disseminated gradually and by the 1970s it emerged as a common cause of hospital acquired infection (HA-MRSA) [107]. The first report of community acquired MRSA (CA-MRSA) comes in 1981 [108] and up until the mid 1990s there was an increasing number of outbreaks [109,110]. What was striking was that the patients involved had not been previously exposed to healthcare settings. CA-MRSA is in most cases a skin infection, however, HA-MRSA can cause life threatening bloodstream infections, pneumonia and surgical site related infections [101].

Centers for Disease Control and Prevention (CDC) statistics show that 2% of the general population are carriers of MRSA [101]. MRSA has been identified as a leading cause of infectious disease in humans and animals [111]. The genetic basis of methicillin resistance has been traced to the staphylococcal cassette chromosome (SCC*mec*) encoding an alternative penicillin-binding protein 2a, which alters the binding efficiency of β-lactam antibiotics on the staphylococcal cell wall [112]. Consequently, cell wall biosynthesis in MRSA strains is not affected even in the presence of otherwise inhibitory levels of β-lactam antibiotics. The first gene to be identified has been *mecA* and has most probably been acquired through horizontal transfer [113], while later on *mecC* [114] was described.

1.5.2 STAPHYLOCCOCAL BIOFILMS

1.5.2.1 GENERAL

S. aureus is a clinically relevant pathogen implicated in a wide range of conditions. Due to the increased occurrence of antimicrobial resistance and its ability to evade the host's immune system, treatment is limited and often ineffective. Treatment failure has been strongly associated with the organism's ability to form biofilms, which have been implicated in recurring and chronic infections [22]. Biofilm-related infections account for approximately 65% of the hospital-acquired infections [101]. Even more important clinically, the majority of biofilm-mediated device-related infections are predominantly caused by either *S. epidermidis* or *S. aureus* [103].

The biofilm, as discussed earlier, has two major components: the cells and their embedding matrix. *S. aureus* can produce multi-layered biofilm structures with the matrix being of variable nature. Earlier studies (naming the matrix glycocalyx or slime) delineated that it was composed mainly of teichoic acids (~80 %) and a mix of staphylococcal and host proteins [115]. However, further analysis identified a specific polysaccharide antigen named polysaccharide intracellular adhesin (PIA) [116], also known as poly-N-acetyl-glucosamine (PNAG). There are two distinct methods for biofilm formation in *S. aureus*: PIA-dependent and PIA-independent [117].

PIA is a positively charged molecule that facilitates intercellular attachment of the negatively charged bacterial cell walls [118]. The *icaABDC* locus is responsible for synthesis (*icaA* and *icaD*), translocation (*icaC*) and modification (*icaB*) of PIA. This locus is present in many staphylococcal species [119] and is associated with enhanced biofilm formation [120]. Regulation of the locus is complex among staphylococci, but it is generally accepted that is regulated by stress conditions (lack of oxygen, extreme temperatures, osmolarity and exposure to antibiotics among others) [121]. Regulatory gene *icaR*, located directly upstream from the *ica* locus, is a well documented negative regulator [122]. Other genes that directly or indirectly have a demonstrated effect on PIA production are: *sarA* [123], *srrAB* [124], *tcaR* [125], *rbf* [126] and *spX* [127]. Table 1 contains a summary of environmental factors that influence PIA expression.

Table 1. Factors that affect PIA expression.

Factor	Reference
Glucose	128
Glucosamine, N-acetylglucosamine	129
Quinupristin-dalfopristin, tetracycline	130
Urea	131
Anaerobiosis	132
Iron limitation	133
High osmolarity, high temperature	134
Ethanol	135

Despite the well-documented importance of the *ica* locus in biofilm formation, staphylococcal biofilms can also form in an PIA-independent fashion. A study investigating the role of *arlRS* (a two-component system known to repress biofilm formation) came to the conclusion that deletion of the above locus resulted in enhanced PIA production [136]. However, when a double mutant was constructed with a deletion of the *ica* locus, biofilm formation was unaffected [136]. Subsequent reports showed that strains with mutation in the *ica*

locus showed no deficiency in biofilm development [137] nor virulence [138]. Fitzpatrick *et al.* [139], working with four clinical MRSA strains, came to the conclusion that there is little correlation between *ica* locus expression and biofilm formation in *S. aureus*. All of the above studies suggested a PIA-independent method for biofilm development that was proposed to be strain-specific.

In *S. epiderimidis*, PIA-independent biofilm formation was mediated by accumulation-associated protein (Aap) [140], while in *S. aureus* biofilm-associated protein (Bap) and Bap-related proteins had the same effect [141]. Both Aap and Bap are located on the cell surface and are involved in cell-to-cell aggregation. These observations pointed towards a proteinaceous cell-to-cell adhesion method for biofilm development that is PIA-independent. Some notable surface proteins with involvement in this mode of biofilm formation are: SasG, SasC, protein A, fibronectin-binding proteins (FnBPA and FnBPB), autolysins (AtlA and AtlE), wall teichoic acids, lipoteichoic acids, cell wall-anchored proteins (such as clumping factors A and B) and the fibrinogen-binding preotein SdrG/Fbe [118].

1.5.2.2 BIOFILM FORMATION

Biofilm formation, as described before, occurs in 5 phases: adhesion, attachment, maturation I, maturation II and dispersal. In this section the specific factors and events in biofilm formation of Staphylococci will be discussed.

Adhesion/Attachment

The initial attachment to an abiotic surface is highly dependent on two factors: the physico-chemical properties of the material and the surface components of the Staphylococci [117]. Surface components involved (but not limited) to the initial phases of attachment are wall teichoic acids [142], lipoteichoic acids [142], accumulation-associated protein (Aap) [143] and autolysins (AtlA [144] and AtlE [145]).

On the other hand, cell wall-anchored proteins are mainly responsible for adherence to biotic surfaces (such as host cells or plasma-coated prosthetic devices). Both *S. aureus* and *S. epidermidis* encode for a variety of microbial surface components that recognize adhesive matrix molecules, allowing adherence to extracellular matrix components, fibrinogen, fibronectin and plasma clots [146]. Among the most important proteins involved in this type of attachment are: surface protein G (SasG) [147], Bap [148], FnbA and FnbB [149], clumping factors (ClfA and ClfB) [150] and fibrinogen-binding protein SdrG/Fbe [118]. Moreover, a collagen binding protein (Can) has been identified in mediating adherence of *S. aureus* to cartilage and collagenous-rich tissues [151].

Some of the above mentioned factors seem to have functions in attachment to both biotic and abiotic surfaces. Notably, autolysins have been shown to facilitate attachment to plastic surfaces [152] and also have binding sites for a variety of host matrix proteins [153].

Maturation & accumulation

After the initial attachment, the cells start producing extracellular polysaccharides, eDNA and other matrix components. The nature of the matrix is dependent on both genetic and environmental factors [37]. Intercellular aggregation is facilitated by PIA production (in the presence of a functional *ica* locus; ica-dependent) and/or by cell wall-anchored proteins (in the absence of *ica* locus; ica-independent). In both cases, the result is the complete encapsulation of the bacterial cells by the matrix, expansion of the cell number in the structure and finally the formation of a multi-layered biofilm.

Dispersal

The last stage in the biofilm mode of life is dispersal, where cells return to a planktonic lifestyle. Dispersal can occur under tight genetic control with the programmed expression of proteases, nucleases and phenol-soluble modulins, that enzymatically degrade matrix components or adhesion molecules [104]. Moreover, quorum sensing molecules have been implicated in mobilizing biofilm cells. Environmental factors include shear stress, corrosion or chemical/mechanical intervention.

1.5.2.3 BIOFILM REGULATION

Accessory gene regulator (*agr*) and staphylococcal accessory regulator A (*sarA*) are among the best-studied global regulatory systems in *S. aureus*. It is generally accepted that they operate in opposing manners, with s*arA* being essential for attachment while *agr* system is mostly involved in dispersal mechanisms. The *agr* system is involved in the downregulation of genes encoding for cell wall-associated adherence factors [154], thus resulting in reduced initial adherence. Repression of *agr* is essential for biofilm formation while induction through auto-inducing peptides is critical for dispersal of mature biofilms [155]. Moreover, *agr* is involved in the regulation of phenol-soluble modulins and nucleases, both implicated in biofilm dispersal mechanisms [156,157]. On the other hand, *sarA* transcripts are shown to be upregulated in biofilm cultures when compared to planktonic cells [123]. Strains with mutations in *sarA* exhibit reduced biofilm capacity [158]. Furthermore, it has been suggested that *sarA* could protect the integrity of the biofilm matrix by inhibiting nuclease and protease expression [159].

The *rpoF* operon in *S. aureus* encodes for the alternative sigma factor B (σ^B) [160]. The role of σ^B in the regulation of biofilm formation is controversial. It was reported that icaR binds to the same region of the *ica* locus promote as σ^B, thus actively downregulating *ica* expression [161]. However, Valle *et al.* [123] showed that in a σ^B deletion mutant biofilm formation was not affected. Others reported that σ^B deficient strains could not produce biofilm [134,162]. σ^B is known to upregulate genes involved in the early stages of biofilm formation (e.g. clumping factors and fibronectin-binding proteins) [163,164] while downregulating a wide range of

proteases involved in dispersal mechanisms [165]. These conflicting reports suggest that the regulatory role of σ^B in biofilm formation may have a strain-specific effect.

Two component systems that are involved in different processes of biofilm formation in Staphylococci are *arlRS* and *lytSR*. Interestingly, the regulatory role of *arlRS* is achieved differently in Staphylococci; in *S. epidermidis* it regulates biofilm formation in a PIA-dependent manner [166], while in *S. aureus* it does so in a PIA-independent manner [167]. The *lytSR* system directly acts on the *lrg*/*cid* operon which regulates cell lysis during biofilm formation [168]. Both systems are known to participate in the events of bacterial autolysis and the release of eDNA, an important component of the biofilm matrix.

1.5.2.4 BIOFILM TREATMENT

Staphylococci are involved in a wide range of infections with biofilm contributing significantly to problematic treatment. Anti-biofilm therapeutics have developed to help treat these biofilm-mediated infections. There have been three different approaches towards an anti-biofilm treatment: development of vaccines, utilization of agents that interfere with essential biofilm factors and development of biomaterials with surfaces that hinder biofilm formation.

There have been several experimental vaccines that showed promising results in animal infection models but it remains to be proven whether they have the desired clinical effect in humans. Two of the most prominent examples were by Kelly-Quintos *et al.* [169], that managed to raise antisera against PIA, and Rennermalm *et al.* [170] who targeted several surface proteins, such as the fibronectin-binding protein. More recently, a conjugate vaccine that contains antisera against PIA and clumping factor A led to accelerated immune response [171]. However, it remains controversial whether vaccination will be an effective measure to control staphylococcal biofilm-mediated infections.

Staphylococcal biofilm formation is multifactorial [104], which makes it challenging (if not impossible) to identify the single factor that is involved in biofilm formation in every staphylococcal infection. However, there have been efforts to target the biosynthesis of factors that seem to be involved in the majority of biofilm-mediated infections. In that case, PIA has been a well-researched candidate. Dispersin B, isolated from *Actinobacillus actinomycetemcomitans*, has the ability to degrade PIA and destroy staphylococcal biofilms [172]. Another anti-biofilm drug -although not biofilm specific- is the peptidoglycan degrading enzyme lysostaphin that is being evaluated for its therapeutic value against biofilms of *S. aureus* and *S.epidermidis* [173].

The development of biomaterials with surfaces that decrease bacterial adhesion has been a very exciting approach on its own right. However, the genetic versatility of Staphylococci results in attachment to the majority of the polymers currently in clinical use. There have been efforts to coat biopolymers with antibiotics or other substances with antibacterial

activity but there was limited success. One of the major problems with such an approach has been the widespread plasmid-borne antibiotic resistance in Staphylococci.

1.6 GUT MICROBIOTA & ITS IMPACT ON HUMAN HEALTH

In recent years, the study of gut microbiota has attracted great research interest. At first, most studies focused on how it affects the regulation of digestive function and satiety [174], while more recently its role in other aspects of physiology is being investigated. There is growing evidence that variations in its composition have an impact on normal physiology and could contribute to diseases ranging from inflammation to metabolic conditions. The existence of an interlinked relationship between host and microbiota is now well accepted [175].

The composition and thus the activity of the gut microbiota is dynamic through the development of the host and has been associated with host genome [176-178], nutrition [179,180] and lifestyle [181,182]. Humans share almost a third of their gut microbiota, while the other two thirds show high levels of individuality [183]. Regardless of the high levels of variations among individuals, it is generally regarded that community stability and species diversity are the key characteristics of a healthy microbiota.

During the initial days of life the gut microbiota is subject to great fluctuation and characterized by low diversity [184]. By the third year of life, its composition stabilizes and in general terms it remains relatively stable to an adult-like profile [185]. The adult microbiota consists of more than 1000 species, more than 7000 strains and is mostly dominated by strictly anaerobic bacteria [183]. Apart from bacteria, the gut microbiota also includes viruses, eukaryotic microorganisms and archaea, but their role in human health is less studied.

The number of microorganisms inhabiting the gut is estimated in between the order of 10^{13} to 10^{14}, a number ten times higher than the number of cells in the human body [183]. The collective number of genes carried by our microbiota exceeds by at least 150 times the number of human genes [183,186] and this has lead to the gut microbiota to be called as "the forgotten organ" [187]. More recent studies have suggested that the growing embryo in the womb might be already exposed to maternal microbes [188], raising questions on the impact of the mother's microbiota on the development and maturation of the intestinal community.

Despite the concept of community stability, there is constant reshaping of the microbial composition driven by complicated dynamic events influenced by diet, life-style choices, disease and antibiotic use. The first three factors have been shown to alter community composition very rapidly [181,182,189], while an antibiotic-related perturbation has a much slower response time, and restoration to a stable community may take weeks or even years to be achieved [190]. More recently, urbanicity was associated with changes in microbial diversity and a profound effect on the gut microbiome functionality [191].

An important -but sometimes overlooked- fact is that there is considerable interpersonal variance in the gut microbiota composition of healthy individuals [192]. Such observation comes with the realization that there are multiple possible combinations that could constitute a healthy gut microbiota, while at the same time stable communities could also be associated with certain disorders [193]. Should we consider the redundancy and pleiotropy of specific microbial members in the overall community composition, it would be rational to think that the functional output of multiple possible combinations could be in theory equivalent or similar.

It has been suggested that the gut-brain axis is a bi-directional route of communication based on neural, hormonal and immunological signals. This communication is considered critical in order for the host to maintain essential functions. The gut microbiota has a profound influence on different aspects of human physiology not only limited to the gut-brain communication axis. It has been associated with pathological conditions that range from inflammatory diseases and obesity to irregularities in behavior and/or physiology that are associated with neurodevelopmental disorders.

The gut microbiota is known to play an important role in the development and functionality of innate and adaptive immune responses [194,195] and in the regulation of gut motility, intestinal barrier homeostasis, nutrient absorption and fat distribution [196,197]. Changes in the gut microbiota have been linked with neurological and psychiatric disorders [198] and more importantly have been shown to modulate brain development and behavior [199]. On the other hand, there are studies demonstrating that stress in adulthood modifies the composition of the gut microbiota [200]. Moreover, other conditions that have been associated with alterations in the gut microbiota are atopic dermatitis [201], systemic lupus erythematosus [202], inflammatory bowel diseases [203,204], type 1 diabetes [205] and multiple sclerosis [206].

The major question that seems to be dominating the field is: does a disease lead to an altered microbiota, or does an altered microbiota directly contribute to the disease? In order to provide a comprehensive answer, prospective and intervention studies are required. Despite rigorous scientific investigations, often more questions arise than answers. For example, a recent study showed that bacterial infection promotes amyloid-β peptide aggregation, suggesting that neurodegeneration in Alzheimer's disease could be linked to host responses to microbial infections [207].

The discovery of the profound effect of the gut microbiota on human health (and disease) has led to the formation of large collaborative projects (MetaHit, Human Microbiome Project, MyNewGut) that have taken metagenomic-based approaches in investigating the cross talk between the gut microbiota and the host. These projects have greatly contributed to our understanding of the importance of the gut microbiota. Furthermore, they have contributed to the association of specific microbial communities with different human diseases, to define environmental factors affecting community dynamics and to the creation of genetic catalogues of reference microbial genes [183,208-210].

Consequently, the modulation of the gut-brain axis -by altering the gut microbiota- has been suggested as a possible process for the development of novel treatments. Three therapeutic approaches have been suggested for gut microbiota treatments: probiotics, antibiotics and fecal microbiota transplantation (FMT). Probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" [211]. A number of studies have proposed the potential of probiotics in the treatment and prevention of diseases. The most commonly employed microorganisms are lactic-producing bacteria such as *Lactobacillus* and *Bifidobacterium*. Despite the fact that their exact mechanism of conferring benefits is not elucidated yet, it has been suggested that they qualitatively change the microbiota composition. Antibiotic treatments alter significantly the composition of the gut microbiota by disrupting its steady state and providing niche for expansion of existing bacteria or establishment of newly introduced ones. FMT has shown promising clinical results in the treatment of recurrent *Clostridium difficile* infections [212] and has been reported to have a beneficial effect in metabolic syndrome [213].

The greatest challenge in the field has been the translation of the vast metagenomic data into a mechanism of biological relevance. The inability of *in vitro* culturing the complete human microbiota hinders efforts to comprehend the complex microbial interactions and the ecological implications of such communities. A possible solution to this has been the effort to identify major metabolites of microbial origin that could modulate physiological processes. This approach would greatly improve our mechanistic understanding of how the microbiota and the host are fine-tuned. Another subject of interest has been the mapping of antibiotic use early in life and the impact it has in shaping the microbiota and its relative functions. The ability to manipulate the microbiota in early life, when its composition is still dynamic, could potentially confer great health benefits later in life.

2 AIMS OF THE THESIS

Understanding the interplay among the host's innate defenses, the virulence arsenal of the pathogen and antibiotic treatment strategies will provide mechanistic insights on antibiotic pharmacodynamics, bacterial pathogenesis and will be invaluable for the development of novel therapeutic interventions.

The overall focus of this thesis has been the study of antibiotic pharmacodynamics. Most of my research has been on biofilms and persister cells with a clear focus on *S. aureus*. My main aim has been to assess whether bacterial physiology has an effect on antibiotic refractoriness.

My specific aims were:

I. The development and validation of a mathematical model based on chemical reaction kinetics that can explain complex patterns of antibiotic action.

II. To study the consequences of long-term culture on antibiotic susceptibility of *S. aureus* using a pharmacodynamics approach, while evaluating a possible link between biofilm formation and persister cells.

III. The in vitro characterization of the antibiotic susceptibility of *S. aureus* biofilms under different incubation atmospheres.

IV. To study the signalling pathways in the gut microbiota-brain axis, and whether perturbations of the gut microbiota by antibiotic treatment early in life could potentially affect the developing brain.

3 PAPERS & DISCUSSION

3.1 PAPER I - CLASSIC REACTION KINETICS CAN EXPLAIN COMPLEX PATTERNS OF ANTIBIOTIC ACTION

The pharmacodynamics of antibiotics have been difficult to predict and have therefore hindered the rational design of antibiotic treatment strategies. Among the major complications in predicting antibiotic activity during treatment are: the post-antibiotic effect, the inoculum effect and the formation of persister cells. All three phenomena are poorly understood and could lead to suboptimal treatment outcomes. In this collaborative study, we clearly show that the kinetics of antibiotic killing, when assessed taking into account the concentrations of antibiotic and microorganism, are predictive of the *in vitro* observed pharmacodynamics. The significance of this study lies in the *a priori* assumption of chemical kinetics driving the interaction between drugs and target with cell death as readout.

We first employed a microfluidic approach, where single *E. coli* cells were exposed to sub-inhibitory concentrations of tetracycline. In these experiments, cells were allowed to grow for 4 h on rich medium, and then subjected to sub-MIC concentrations of tetracycline for 16 h and their replication was monitored in the post-antibiotic phase for 4 h. A model was used to fit this data and it succeeded in reproducing the observed pattern of post-antibiotic effect, suggesting that the kinetics of antibiotic-target binding are sufficient to explain this effect. Moreover, our model also predicted that slower bacterial growth rates are associated with a stronger post-antibiotic effect.

We then sought to investigate the impact of density-dependent antibiotic effects. In order to test the predictive power of the developed model, we used data from batch cultures of *E. coli* and *Vibrio cholera* exposed to five and six antibiotics, respectively. From the batch cultures, we concluded that antibiotic activity reduced with increasing bacterial density in all bacteria-drug combinations (albeit at different extent). Our model -based on the assumption that antibiotic molecules are not an unlimited resource- managed to reproduce this effect.

Previous studies trying to describe the declining killing efficacy observed in *in vitro* time kill assays have attributed the invariable pharmacodynamic profile to cellular persistence and a stochastic switch. We found that of the five antibiotics we tested, there was a strong correlation between persistence and antibiotic concentration for streptomycin and ciprofloxacin. *In vitro* time kill experiments validated these results, and we additionally used published clinical data from *in vivo* treatments to investigate further the validity of our model. Again, we noted concentration-dependent persistence. This demonstrated that our model, based on chemical kinetics alone could account for multiphasic kill curves as well as concentration-dependent persistence.

The developed mathematical model was able to predict the challenging phenomena described above and provide an alternative hypothesis for why antibiotic killing slows down over time; declining concentrations of free target and/or available antibiotic show strong correlation to the experimentally observed situation for all drugs and species considered. In addition to this,

we showed that the inoculum effect was also predictable for different drugs and species, as well as the duration of the so-called post-antibiotic effect.

3.2 PAPER II - STATIONARY PHASE PERSISTENCE IN STAPHYLOCOCCUS AUREUS IS DRIVEN BY BIOFILM FORMATION

While much work has been done on the susceptibility of exponentially grown cultures of *S. aureus*, stationary phase has remained underexplored. The effects of antibiotics on these older, denser and slow growing cells are quite diverse when compared to those of their exponentially growing counterparts. In this study we seek to determine whether the physiological state of the bacteria affects their susceptibility to six clinically relevant antibiotics and to what extent.

To ascertain this, we examined the contribution of media and stationary phase physiology to *in vitro* estimates of antibiotic efficacy. Having established that extended culturing times lead to gradual alkalification of the medium, with adverse effects on antibiotic efficacy, we opted to perform our time kill assays on fresh medium. Moreover, we adjusted the bacterial densities of the differentially aged cells and exposed them to antibiotics at similar densities to avoid any inoculum-related effects.

Differentially aged cultures (exponential, 1-, 3- and 7-day old) were exposed to six clinically relevant antibiotics (ciprofloxacin, daptomycin, gentamicin, linezolid, oxacillin and vancomycin) in order to study whether culture age has an effect on antibiotic refractoriness. Our results clearly demonstrated that older cultures were significantly more refractory than young ones to all six antibiotics. Moreover, the observed refractoriness showed strong age-dependency.

We then tested for growth-dependent effects on susceptibility, since bacterial susceptibility is tightly intertwined with metabolism and growth kinetics. To that end, we used carbonyl cyanide m-chlorophenyl hydrazone (CCCP), a known proton motive force decoupler. We reported that for five (linezolid excluded) of the antibiotics, CCCP-treated cultures were significantly less susceptible when compared to their respective control cultures. Interestingly, linezolid exhibited increased efficacy in the presence of CCCP.

Long-term culture resulted in increased biofilm formation. Consequently, the contribution of biofilm formation to the relative refractoriness was evaluated. Biofilm-residing cells were released by sonication and exposed to the same panel of six antibiotics. As with planktonic cells, refractoriness increased with culture age. Notably, we observed significantly higher refractoriness of these erstwhile biofilm-resident cells when compared to their planktonic counterparts.

We investigated whether a flux between the planktonic and biofilm mode of life existed in our experimental system. We competed our wild type (WT) strain and a ciprofloxacin-resistant (R) derivative of it. We performed biofilm formation assays and then removed

planktonic cells introducing an "invasive" population, while following dynamic changes in the ratios of the WT and R cell numbers. We thus confirmed the existence of a constant flux of cells between the biofilm and planktonic mode of life.

Having ascertained that cultures were increasingly more refractory with age, we sought to investigate whether morphological changes -that have been associated with antibiotic refractoriness- were evident. From transmission electron microscopy images we analyzed cell size and morphology, cell wall thickness, and replication frequencies. In brief, we demonstrated that older cultures were enriched in smaller cells with thicker cell walls. Cells from younger cultures exhibited signs of active metabolism, while older cells were less metabolically active (solely based on ribosomal content) and had more condensed chromosomes.

Next, we explored the contribution of 23 genes that are involved in global regulatory networks, metabolism, virulence, cell division, cell wall biosynthesis and biofilm formation. In brief, we reported gene expression patterns (*agrA*, *mgrA*, *sasG*, *icaA*, *sarX*, *rot*) consistent with a regulatory network that favors attachment in long-term cultures.

Overall, we showed that refractoriness is progressive, with older cultures being less susceptible than younger cultures. Flux from biofilm to the planktonic compartment resulted in sustainably refractory cells. We identified biofilm seeding as the primary cause of this refractoriness. Our data supports an alternate hypothesis as to the origin of persister cells, e.g. erstwhile biofilm-resident cells with retained general antibiotic refractoriness.

3.3 PAPER III - ANAEROBIC CULTURE CONDITIONS AND THEIR EFFECT ON THE SUSCEPTIBILITY OF STAPHYLOCOCCUS AUREUS BIOFILMS

Biofilm-related infections account for approximately 65% of the hospital-acquired infections, according to the Centers for Disease Control and Prevention. Biofilms have also been strongly correlated with chronic and latent infections. *S. aureus* is a prominent biofilm former and its ability to cause infection has been associated with biofilm formation. Biofilm development is Staphylococci has been ascribed to two mechanisms: ica-dependent and ica-independent.

The molecular basis of the ica-dependent mechanism is the transcription of the *icaADBC* locus, which is responsible for the production of polysaccharide intracellular adhesin, considered a critical component of the biofilm matrix. On the contrary, in the case of ica-independent mechanism, other surface-related adhesins facilitate biofilm formation (e.g. *sasG*).

In this study we sought to investigate the role of incubation atmosphere on antimicrobial susceptibility of *S. aureus* to three clinically relevant antibiotics (ciprofloxacin, daptomycin and gentamicin). In paper II, we reported that biofilm-released cells were more refractory than their planktonic counterparts. Here, we exposed biofilm-derived cells of two major

adhesin knockout mutants (Δ*icaA* and Δ*sasG*) and recorded their susceptibility under aerobic and anaerobic conditions.

We first assessed biofilm formation on all three strains (WT, Δ*icaA* and Δ*sasG*) using a microtiter plate assay. We concluded that under anaerobic conditions there is no significant difference on biofilm formation. Interestingly, we reported that Δ*sasG* forms significantly more biofilm (23 %) under aerobic conditions.

Since crystal violet staining is non-specific (binding both matrix and cell components) we decided to directly assess the cell density in biofilms. To remove the biofilm-residing cells from their embedding matrix we used sonication. Under aerobic conditions, we reported that all three strains had similar cell densities. On the other hand, under anaerobic conditions, Δ*icaA* had more than three times more cells embedded in the biofilm structure.

We then exposed the released biofilm cells to our panel of antibiotics. In brief, we reported that aerobic exposure to ciprofloxacin had no effect on the susceptibilities of the three strains. However, under anaerobic conditions all strains exhibited enhanced survival. Daptomycin exposure under aerobic conditions sterilized WT cultures. Under anaerobic conditions, daptomycin exposure was less effective but at a similar level among all strains. For gentamicin we reported the highest differences between aerobic and anaerobic conditions (2,5 log better survival under anaerobic conditions).

In conclusion, we reported discrepancies between biofilm assessment by crystal violet and direct enumeration of cells residing in the biofilm. More importantly, we showed a significantly decreased susceptibility of *S. aureus* biofilm cells under anaerobic conditions to three clinically relevant antibiotics.

3.4 PAPER IV - THE BACTERIAL PEPTIDOGLYCAN-SENSING MOLECULE PGLYRP2 MODULATES BRAIN DEVELOPMENT AND BEHAVIOUR

A growing number of studies have revealed that the gut microbiota can modulate brain development and behavior; however, our understanding of the molecular mechanism(s) governing these interactions is still limited. In this collaborative study, we investigated the translocation of bacterial peptidoglycan from the gut and its impact on the developing brain.

First, we set to test whether peptidoglycan could be translocated in the blood under normal conditions. We found that the peptidoglycan levels in the serum of germ-free mice were significantly lower when compared to specific pathogen-free mice. Then, we investigated if bacterial peptidoglycan could cross the blood brain barrier and showed evidence of such translocation. The next step was to investigate the levels of peptidoglycan in three different brain regions (prefrontal cortex, cerebellum and striatum) from mice at various postnatal ages (1, 3, 5, 7, 14, 21 and 60 days). We reported an age-dependent increase in peptidoglycan levels for all three tested brain regions. No significant differences were found between male and female mice, suggestive of sex-differences not being a confounding factor.

Having ascertained that peptidoglycan from the gut microbiota can be translocated through blood circulation to the developing brain, we sought to investigate the expression profiles of peptidoglycan sensing molecules in the striatum by qRT-PCR during these early stages of development. We focused on the expression profiles of two specific pattern-recognition receptors, which have been reported previously to recognize peptidoglycan; peptidoglycan-recognition proteins (*Pglyrp1*, *Pglyrp2*, *Pglyrp3* and *Pglyrp4*) and the NOD like receptors (*Nod1* and *Nod2*). Moreover, we investigated the expression of Toll-like receptor 2, *Tlr2* (that recognizes peptidoglycan and other components of microbial origin) and peptide transporter 1, *PepT1* (that has been shown to efficiently translocate peptidoglycan fragments).

We reported that all four peptidoglycan-recognition proteins were expressed in the developing brain during specific temporal windows of postnatal development. *Pglyrp1* expression gradually increases reaching a peak at 14 postnatal, while afterwards decreases to adult levels. *Pglyrp2*, *Pglyrp3* and *Pglyrp4* on the other hand, were significantly more expressed during the first days of life and showed a steady decline in expression with age. A direct comparison of the expression profiles between male and female mice showed significant sex differences. The gene expression levels of *Nod1* were significantly lower early in life, when compared to adult and had a peak at 21 days postnatally for both sexes. On the contrary, *Nod2* gene expression showed a gradual increase with age for both sexes. *Tlr2* and *PepT1* mRNA levels were highest at the first day of life and declined over time.

The gene expression of the all target genes was also assessed in the prefrontal cortex and cerebellum. For *Pglyrp2*, *Pglyrp3*, *Pglyrp4*, *Tlr2* and *PepT1*, the expression profiles showed a similar pattern. Interestingly, more profound sex differences were noted in the prefrontal cortex, followed by the cerebellum and the striatum. The expression of *Pglyrp1*, *Nod1* and *Nod2* showed brain regional differences and strong sex differences. In summary, the above results indicate the existence of an age-, region- and sex-specific manner by which the gut microbiota can influence brain development.

We then decided to evaluate whether manipulation of the gut microbiota could influence the expression profiles of the peptidoglycan sensing molecules in the developing brain. Two experimental approaches were employed: germ-free mice and pregnant mice that were exposed to ampicillin during the last week of gestation and first 3 days post-partum. A comparison of the expression profiles between the two models (and their respective controls) indicated that several peptidoglycan-sensing molecules are responsive to perturbations of the gut microbiota.

Furthermore, the specific role of Pglyrp2 was investigated, due to its specific function, high expression levels early at life and sensitivity to microbiota manipulations. Our results suggested that its deletion changed synapse-related gene expression and exhibited a strong sex-dependent variance. Juvenile *Pglyrp2* knockout mice were subjected to an array of tests to assess exploratory activity, anxiety and social behavior. Collectively the results from the behavioral studies signified that Pglyrp2 modulates the development of social behavior.

In summary, this publication provides evidence that peptidoglycan sensing by specific pattern recognition receptors could be one of the signaling pathways mediating the communication between the gut microbiota and the developing brain.

4 FUTURE PERSPECTIVES

Microbial research has long been focusing on single species, with most of the knowledge we have today coming from mono-species studies. Though convenient for *in vitro* studies, bacteria rarely ever exist as single species in natural settings. Different species with completely different metabolic profiles and/or lifestyles can reside together, with biofilm seeming to be the most preferred mode life of a given polymicrobial community.

The implication of biofilm-related infections, as discussed earlier, shows that they are one of the major impediments to healing and enables the chronicity of such infections [214]. Within an infection, biofilm formation is an important barrier to an effective treatment. To complicate this scenario further, though unsurprising, there is speculation of persister cells also residing in biofilms [78,215,216].

Resistance of biofilms to antibiotics has at times been attributed to poor penetration of the drug and the inability to physically access all cells residing in its depth [217]. More recent studies, however, provide strong evidence that biofilm matrix does not explicitly restrict diffusion of antibiotics through it [218]. It has alternatively been suggested that the matrix may instead act primarily as a barrier for larger components of the immune system such as macrophages and neutrophils [36,219].

The understanding that established infections form a rather complex and dynamic ecological milieu is becoming more prevalent. This milieu would often include different species, growing under suboptimal conditions and constantly competing for space and resources. Adding biofilm formation and an intrinsic rate of persister formation amongst the different species in the community only serves as an approximation to the problem. Superimposed upon all the above is the host immune response (with its interacting components), which is yet to be accounted for. Taken together, the scenario described above reveals a need for a deeper understanding of these communities in the site of infection. Only when the forces that govern the ecological relation(s) of the interacting species are elucidated, we can proceed and discuss a successful therapeutic regimen.

"The possession of knowledge does not kill the sense of wonder and mystery. There is always more mystery"

Anaïs Nin

5 ACKNOWLEDGEMENTS

It has been a long journey and I can only be grateful for all the people I have met along this experience. This thesis wouldn't be a reality without everyone mentioned below. Each and every one of you helped (in their own way) and made it easier for me to reach the finishing line.

First to my main supervisor: **Klas**. What a ride right? There were good times and there were rough times. However, we managed to work problems through and reach a state of understanding. Working under you is a constant challenge that either breaks people or makes them try harder to prove themselves. I know you don't like advice but I will give you one! Inspire your students and you'll be amazed at what they will give back to you. I hope in a few years time, when you tell people of your first PhD student, you remember the good times.

To my co-supervisors: **Andreas**, **Rochellys** and **May**. I am very glad to have been able to co-publish with everyone and each of you! **Andreas**, thank you for all your advice and assistance. I want to express my gratitude for "adopting" me when Klas was on parental leave and being supportive and always trying to solve every problem that came up. You were always pro-active in finding solutions and offering help, I am happy and grateful we got the chance to work together. **Rochellys**, I am truly short of words (and appreciation) here. First of all, thank you for sharing your knowledge with me and giving me the chance to work with you in the lab. I know the fields of neuroscience and microbiology have "evolved" separately, but you saw a convergence point and this odd cross-disciplinary view of yours is what amazes me. You have always been supportive, patient and understanding. I have truly enjoyed discussing science and life in general with you. I want to express my gratitude for your help and support when I needed them the most. I only wish we had had the chance to collaborate in more projects. **May**, thank you for accepting to be my co-supervisor even before formally meeting me. We may have not interacted as much as originally planned, however, whenever I needed anything you were there for me. Thank you for that.

To my group members (former and present). First (before everyone else!), **Eva**; I am again short of words here! You helped and inspired me more than any person I have ever worked with. There were times we could understand each other with just a look. I think the saddest day in my lab life was when you told me that you are leaving the group. I was a bit upset (why deny it!) at first, but only for a few weeks. Deep inside I was happy for you! I still remember your first week in KI, when you asked me if I take coffee breaks and I replied something like: "I don't usually take breaks". You had the funniest/confused face ever! You have been an amazing lab mate, understanding and caring. I was amazed at your resolution when faced with challenges. You have always tried to improve yourself and everyone around you. You are by far and with no doubt the one only person I would love to be lab mates forever! You will also forever be my qPCR mentor!!! **Paulina**, what a force of nature you are! I think every lab needs a crazy (only with the good meaning) Polish person like you in their roster. You work hard and party hard. I am extremely happy to have met you and worked with you. You are smart, fast learning and a delight to work with. You always have a

smile in your face and problems do not exist for you. You sometimes thought your jokes were inappropriate, but I tell you I did laugh a lot! You are a special person and I am positive you will find your way. I have faith in you! **Ymke**, thank you for being such a fantastic colleague. You were the second PhD student to join the group and I still remember the positive impression I got after your final interview with Klas. You were thrown in deep water but you learned very fast how to "swim". You organized most of the lab logistics making everyone else's life much easier. I enjoyed working as much as having off-work lunches with you. I think both Eva and I were worried about you in the beginning but you proved us wrong! I hope the future will bring what you want of it ;) **Björn**, it has been a great experience to work and talk with you. We discussed both work and life and I was amazed with your views. You are a very realistic person and I think that's the quality I liked the most about you. You helped me with my bioinformatic explorations and I only wish I had more time to learn from you. I will always appreciate those long Friday lunches with the group! It is such a pity you are not around anymore. **Karolina**, always with a smile on your face, you made every day in the lab a little sunnier. I still remember when you tried to pass on your bioinformatics knowledge in 30 minutes on a late Friday evening. I hate to admit, most of it went over my head... Thank you for trying to establish fika with homemade cakes! It was fun working with you in the lab and I hope the high school microbiome project takes off. **Omneya** and **Pilar**, the newest additions in the group, I wish you good luck with your projects. We met in a very stressful point of my PhD (finalizing manuscripts and balancing home life with a newborn). I hope I haven't been too mean to you! I have enjoyed chatting and hanging out in the kitchen with both of you. **Daisy**, it has been an absolute delight sharing the lab with you. I still cannot understand where you get all this never-ending energy! I think you are the hardest working person I have ever worked with. You always had a smile on your face and you made friends in seconds. You were also the person to introduce me to the 12 pm lunch, not because I was hungry but because it was "socially proper". **Joanna**, by far my favorite master student! I had great laughs with you in the lab and I really missed you when you left! **Eric**, you introduced me to microfluidics. I only wish this project had took off. You were in the group for a short time but I still miss the long philosophical conversations we had. **Joanna R**, I certainly enjoyed helping you set up your sequencing runs. You are a hard working person with resolution and great determination. Problems are only there to be solved! I enjoyed our long discussions in the office and the kitchen. I know your defense is also near and I believe you'll do great. **Ellie**, my colony-counting angel! I think I would be still counting plates without you! I really really appreciate your help! **Emma**, the most formally dressed young person I have ever met. I will never forget the long (and outrageously fun) discussions with the rest of the gang (Paulina, Björn and Ellie). It has been very fun to have you in the office.

To all the people in the Medical Nanoscience Center where I started my PhD journey (if I don't write names it is certain I won't be forgetting anyone!). Thank you all for accepting me in the "KI family"! Special mention to Bjorn Hogberg's group, such a loud and happy crowd! **Alan**, **Ferenc**, **Joao**, **Erik**, **Giulio**, **Esther**, **Cosimo** and **Ioanna**, you adopted me in every

social event. It was great fun to be around you all! Those late after work evenings playing games are among my nicest memories. **Ben**, the first microbiologist to join the corridor after me, it has been a great pleasure (and relief) discussing my experiments with you. You always had all kinds of suggestions to improve every experiment I had trouble with. Discussing science over a beer (or two) has never been more fun.

To all the people in Stockholm University, with whom I spent the last stretch of my PhD. **Sunil**, **Hanna**, **Gabriela**, **Raquel**, **Sara**, **Jacob**, **Sushil** and **Nele**. I thank you for all the exciting presentations and inspiring discussions. You all made my transition to SU so much easier! All of you helped me in various ways (showing me equipment, explaining routines, critically discussing my results, and so much more!) and I am very grateful to each and every one of you. **Sunil**, the lab wizard. Every singe time I asked you something, you always came up with a comprehensive answer. I believe you will be a very successful group leader. **Hanna**, you were always with a big smile and eager to help. **Gabriela**, a nature enthusiast that always had a positive and supporting word to say. **Raquel**, sorry I "stole" your desk! I will particularly remember your relevant feedback after every presentation. **Sara**, thanks for all you help and all the discussions we had during lunch. **Jacob**, despite the little time we interacted you were always helpful. **Sushil**, thanks for always having a smile in your face whenever we met. **Nele**, I am particularly grateful for all the discussions we had while leaving late from the lab. I might have missed three or four trains on the process, but it was simply so effortless discussing with you. From life to science and back to life I believe we thought alike on so many levels. **Themis**, a stressed and scared master student at first, a confident PhD candidate now. I am happy you decided to apply for that position. I will always remember the fun discussions we had when we bumped into each other in the corridor. You made every day in the lab something to look up to; I expect great things of you! **Yeneneh**, you have been the nicest person I have met in a long time. Always with a smile, never complained about a single thing and full of hope. Thanks for being such a great colleague and caring about everyone! A very special mention to **Beatriz**, the superhero of DMB. You are the one that keeps everything together around there. You solved every problem in a blink and seemed to anticipate anything we could ever need. Always happy, with a big smile in your face and a kind word for (almost) everyone. You are surely going to be missed! I will kindly remember our conversations in a mix of Spanish, Swedish and English. You are an angel!

To other people I worked together with: **Pia**, thank you so much for giving me the opportunity to work with you. Thanks for the fun, informal and productive meetings in KI, it was great to have your support. You are a kind and hard working person. When you discuss your work you have a spark that captivates. It is inspiring to attend your presentations. Thank you for all the help while preparing my defence. **Tim**, it has been a great pleasure to collaborate with you. Exchanging knowledge in microbiology and neuroscience has been fun. Thank you for your help and support when I needed it. Best of luck with your future plans. **Claudio**, it has been fun to work together. Discussing movies and games while doing science

is the best past time for long incubation periods. Thanks for buying me a beer the day I got stranded in Stockholm. I know your defence is soon so I wish you the best of luck!

To my friends: **Belen**, I remember meeting you in the queue of a hostel in the distant 2010. Since then you have been the third most important member of my Swedish family! I cherish all the trips we took, all the alcohol we consumed, all the songs we sung (with our terrible voices), all the videogames (TAKE COVER!!!) we played and possibly every moment we spend together. Special mention to PPP nights, especially the ones with the bad (very bad) movies. We lived together, we partied together, we cried and laughed together, we shared special moments. You have my unconditional love! Amigos para siempre (sung by Los Manolos). To my two favorite Americans: **Olof** and **Lexi**. I wish I could introduce you to each other one day. I envision epic and endless conversations over every possible topic. **Olof**, you have been a great friend since the beginning of our master studies. I cannot even recall how we met. However, you are a person I admire and think greatly of. I was quite sad when you first moved to Lund and later to Korea. We shared beautiful moments with great conversations, epic laughs and endless dinners. I wish one day we live close enough so you can take little Alex to forest adventures. Miss you a lot! **Lexi**, I don't know if I have ever told you, but I think you are a very special person. I am proud of you for choosing to do something that truly makes you happy. I will always smile with your "call me" gesture (and NO there is no other meaning in it!). I deeply enjoyed our long conversations and the Belgian beers we shared. You were a great addition in the office and I hated it when you left. I am very happy you enjoy were you are now. I sincerely hope we keep in touch. **Magda & Tom**, I will never forget all the special moments we spent during our master time. I sincerely missed you when you moved to Stockholm. I believe you are both great people and I hope we keep in touch for the years to come. **Heidur & Fabio**, best of luck with the newest member that is about to join the family! You are both great people and amazing hosts. I really look forward to common play dates. **Edda & Ari**, thank you for being so close friends to Arancha and me. Little Björk is lucky to have you as parents. I hope we continue with our Sunday brunch tradition for the years to come. **Enrique & Lena**, I hope you move to Uppsala and we get to spend more time together. You are both very fun people to be around! Enrique, I will always remember fondly the hidden energy that you channeled when you tried to rip off gift-wrappings during the Christmas games! **Maria & Tonia**, we met through friends that are no longer part of our lives. However, this did not stop us from becoming close friends. Thank you for taking care of our plants and little Alex (more occasions will appear in the future in case you were worried!). We have to agree on a new poison of choice, since this will be the last year with tsipouro reserves. Maria, you are a star! I couldn't be happier for you! **Stavros & Valia**, thank you for being such good friends! We have enjoyed multiple game nights and meals. And now a special rule: carrying the thesis with you in game nights gives you each a permanent +2 in munchkin fights and reciting a part of it by heart a +5 (usable only once!). Looking forward to summer barbeques! **Eleniki**, that will be your name forever now in my book! We shared some great dinners, laughs and tears. We surely missed you when you left for Ireland and then to Canada. I hope the future will find us in a closer geographical

proximity. **Vasso**, even though you returned to Greece we still miss you here! Best of luck to you and little Stratos. The munchkin gang is missing you! **Patricia & Arnold**, I think we first got together due to our common love for board games, but we kept hanging out because we enjoyed each others company. You are very dear friends and a great couple. If we are lucky, one day we might live in the same place and our kids get to become best friends! A kiss to Miri and Alexander. **Mikaela**, my first ever Swedish friend! We met in a crazy corridor and we partied a lot with that crazy bunch. You will forever have a special place in my heart. I sincerely believe that you and Stian are an awesome couple and I look forward to your wedding. **Elina A**, we met in Guides a million years ago! I remember I had just started studying biology and you were super interested in following the same path. I am happy you did and I am even happier you managed to do so well! I remember one day I was at your place and your mother (that had just met me) said that I had been you mentor and inspired you to follow that path. I felt utterly embarrassed and proud at the same time. I don't think I did influence your choices that much but it felt special to hear such kind words. Best of luck in your future ventures. **Elina & Panos**, I could write pages over pages for both of you, but I will try to keep it short and express my gratitude in person and uncensored! You have been my chosen family since the beginning of times. Elina, I could recite endless events from our lives that I am grateful you were there to support me or offer a comforting hug. We literally grew up together and even though we hated each other at our teens, you are the only person (that is no direct family) that I care so deeply about. You have a heart of pure gold and I can only feel lucky to call you my friend. You are a special person and you deserve the best! Nevertheless, I will never ever forget you did not invite me to your wedding. Never. Ever. Πάνο, να ξέρεις ακόμα απορώ πως την αντέχεις αυτή τη φίλη μου! Είσαι ήρωας!!! Έχετε και οι δυο την άπειρη αγάπη και υποστήριξη μου. Μπορεί να είμαστε (σχετικά) μακρυά αλλά είστε πάντα δίπλα μου! Είσασταν το καλύτερο δώρο που πήρα ποτέ! Περιμένω με ανυπομονησία τα ανηψάκια!!

An exceptional THANK YOU is in order for a special few that hold a very dear place in my heart. I know I haven't been (lately) the best friend to all of you, but you have all been with me in key moments of my life and nothing will make me forget that. **Δημήτρη** Γιαννάκη, άρχοντα των ελληνικών δρόμων. Γνωριστήκαμε το 1997 (πέρασαν κιόλας 20 χρόνια;;;) στον οδηγισμό. Αν θυμάμαι καλά γίναμε φίλοι λόγω της κοινής «συμπάθειάς» μας για το ΤΤΞ. Μετά απο αυτό μοιραζόμασταν για πάντα την ίδια σκηνή. Δεν ξέρω αν στο είπα ποτέ αλλά ήσουν ο καλύτερος φίλος των παιδικών και εφηβικών μου χρόνων. Κάναμε τρέλλες (θυμάσαι το ένα ποτάκι στην Πάτρα με το σμαρτάκι; το σιχαμερό πιτόγυρο στα Καλάβρυτα; το ταξίδι στη Θεσσαλονίκη με το φορτηγό; τις βόλτες στο Ξυλόκαστρο; τα καρναβάλια στην Πάτρα;), κυνηγήσαμε κορίτσια σε όλη την Ελλάδα και ζήσαμε στιγμές ανεπανάληπτες. Γελάσαμε, κλάψαμε και χτίσαμε μια φιλία που για μένα είναι αξέχαστη. Θυμάμαι τους επικούς καβγάδες για τη διαφορετική μουσική που μας άρεσε. Προσπαθώ να θυμηθώ αν ποτε καβγαδίσαμε για κάτι άλλο και δεν μου έρχεται τίποτα. Μπορεί να χαθήκαμε όταν έφυγα για τη Σουηδία αλλά όταν σκέφτομαι τους αγαπημένους μου πίσω στην Ελλάδα είσαι πάντα εκεί! Καλά και ασφαλή χιλιόμετρα φίλε! **Πέτρο** Κανέλλο, φίλε καλέ, φίλε τρελλέ και

τρισμέγιστε ταξιδιώτη! Γνωριστήκαμε σε ένα αμφιθέατρο του μαθηματικού ίσως και στην πρώτη διάλεξη ποτέ. Έχοντας 2 χρόνια παραπάνω ήσουν ο «παππούς» της παρέας μα ήσουν και η τρελλή καρδιά της. Άπειρες βόλτες με το φοκούλι, σαλατούλες στα Friday's, συνέδρια-εκδρομές σε όλη την Ελλάδα. Μπορεί να μέναμε σε αντιδιαμετρικές περιοχές μα αυτό δεν υπήρξε ποτέ εμπόδιο να βρεθούμε και να διασκεδάσουμε. Η φοιτητική ζωή μου ήταν πολύ καλύτερη εξαιτίας σου. Ταινίες, δείπνα, παντομίμες (λούκου μάδες με μέλι), συζητήσεις, σουβλάκια (πολλά σουβλάκια), ποτάκια σε μια καλοκαιρινή Αθήνα, βόλτες, κρέπες, ταξίδια, σινεμά. Τι να πρωτοθυμηθώ!!!! Είσαι ένας σπάνιος άνθρωπος και παρόλες τις δυσκολίες πάντα είχες ένα τεράστιο χαμόγελο και μια τεράστια καρδιά. Χαίρομαι που πήγες το πρώτο σου ταξίδι στο εξωτερικό μαζί μου. Εκείνο το 3ήμερο στο Λονδίνο ήταν ίσως το πιο αυθόρμητο ταξίδι όλων των εποχών (με την ίζιπεφτ!!). Μερικοί άνθρωποι δεν είναι κοντά σου αλλά πάντα μες στην καρδιά σου. Ελπίζω να καταφέρουμε επιτέλους να συναντηθούμε κάπου στην Ευρώπη! **Αμαλία** Κουζώφ, η αγαπημένη μου grande ψωνάρα όλων των εποχών. Γνωριστήκαμε πραγματικά σε μια κατασκήνωση στις Πρέσπες, αν και για να πούμε την αλήθεια είχαμε γνωριστεί μια χρονιά νωρίτερα σε ένα 3ήμερο για στελέχη (λίμνη Πλαστήρα;). Για κάποιο λόγο όλοι οι κοινοί μας γνωστοί δε σε συμπαθούσαν, μα όταν γνωριστήκαμε κολλήσαμε για τα καλά. Μπορεί στην αρχή να με ζάλισες με το «που είναι η κατασκευή για το πλύσιμο πιάτων» αλλά εκείνο το μαγείρεμα με καμμιά 50αριά μπούτια κοτόπουλο θα μείνει στην ιστορία (θυμάσαι το κριτσικάκι;). Δε θα ξεχάσω ποτέ εκείνη τη βραδυά μετά από ένα ατελείωτο συμβούλιο, αρχίσαμε να μιλάμε και μας βρήκε το ξημέρωμα σε ένα παγκάκι με τις καρδιές μας ανοιχτά βιβλία. Δε θυμάμαι την παραμικρή λέξη απο εκείνη τη βραδυά αλλά θυμάμαι εμάς αγκαλιά να βλέπουμε την ανατολή πάνω από τη λίμνη σαν να έχουν ξεφορτωθεί όλα τα βάρη από την ψυχή μας. Αξία ανεκτίμητη! Να ξέρεις ακόμα κουβαλάω μαζί μου την αφιέρωσή σου από εκείνη την κατασκήνωση. Ελπίζω πλέον μετά από ένα χαλαρό ποτάκι να ξέρεις πως να φορέσεις το μπουφάν σου. Σε αγαπώ! **Μαρία Ιω** Ακαλέστου, θα μπορούσα εύκολα να γράψω σελίδες για σένα. Σπάνια στη ζωή συναντάς ανθρώπους με τη δική σου σοφία. Ειλικρινά στύβω το μυαλό μου να θυμηθώ που και πως γνωριστήκαμε. Νομίζω το είχαμε συζητήσει στο παρελθόν και καταλήξαμε οτι είχαμε γνωριστεί στης Τσιμπούκη (της Ειρήνης!), σε μια εκπαίδευση, σωστά; Θυμάμαι τις βραδιές στη Δροσιά με σουβλάκια και ταινίες, με μουσική και κρασί, με συζητήσεις μέχρι το ξημέρωμα. Δεν ξεχνώ πως για πρώτη φορά είδα όλους τους τίτλους τέλους ταινιών με κάποιον άλλο. Δεν ξεχνώ εκείνο το φρικτό σιντί με το πιάνο. Μου έδωσες βιβλία, μουσική και σοφία (τα καλύτερα δώρα που πήρα ποτέ!). Κατά καιρούς μου έδωσες σημειώματα με αποφθέγματα, να ξέρεις τα έχω όλα μαζί μου στη Σουηδία. Νομιζω γνωριστήκαμε σε κομβικά σημεία της ζωής μας (*...και ξαναβάλεις τις ρόδες μου σε ράγες και εγώ αρχίσω να κυλάω, να κυλάω, να κυλάω ξανά)*. Όταν ένιωσα χαμένος ήσουν εκεί να μου κρατήσεις το χέρι και με ενα γλυκό χαμόγελο και λόγο πήρες τον πόνο μου μακρυά. Μου έδωσες τόσα πολλά και για πάντα θα αναρωτιέμαι αν κατάφερα να σου δώσω κάτι πίσω. Η αγάπη είναι πολύ μικρή λέξη για να χωρέσει αυτό που νιώθω για σένα.

To a special microbiologist and dearest friend, **Despoina** Lymperopoulou. I never got the chance to completely express my gratitude to you, so I choose to do it here. You were my first true mentor. You introduced me to microbiology and your passion in your work made me love it too! You taught me so many things that without them I wouldn't be where I am now. Skills I learned from you are still with me. You have been the best teacher a student could have. I am deeply grateful for that!

To my Spanish family: **Arancha**, **Ramon**, **Adrian**, **Berta**, **Iñigo** y **Guille**. Hemos pasado muchas estupendas vacaciones juntos. Siempre intentais enseñarme cosas nuevas y me llevais a un montón de sitios. Si soy honesto, tengo que decir que muchas veces me habeis tratado incluso mejor que mi propia familia! Arancha siempre habla sobre vosotros con palabras de afecto y cariño. Me parece increíble lo cercanos que sois entre todos y cómo podeis pasar horas y horas hablando por Skype. Arancha os quiere a todos incondicionalmente y sois una gran parte de su vida. La distancia entre vosotros pesa enormemente sobre ella.

To my family in Greece. Στην οικογένειά μου. Στον παππού μου **Χρήστο** και στη γιαγιά μου **Διάνα**, χωρίς εσάς δε θα είχα ποτέ καταφέρει τόσα πολλά. Με στηρίξατε, με συμβουλέψατε και με αγαπήσατε άνευ όρων. Με υποστηρίξατε στα όνειρα μου και με προσγειώσατε όταν πετούσα στα σύννεφα. Είσασταν, είστε και θα είστε οι «κολώνες» της ζωής μου. Χωρίς εσάς δε θα ήμουν ο άνθρωπος που είμαι σήμερα. Στη μητέρα μου **Αθηνά**, που θυσίασε τόσα πολλά για να κυνηγήσω το ονειρό μου. Η αγάπη σου και υποστήριξή σου με βοήθησαν να φτάσω ως εδώ. Πάντα είχες την υπομονή να ακούς τις γκρίνιες μου και να με συμβουλεύεις να δρω με λογική και όχι συνάισθημα. Είσαι πάντα δίπλα μου. Στη θεία μου **Άρτεμις**, μπορεί να είσαι στην άλλη άκρη της γης αλλά πάντα ήσουν στο πλευρό μου. Στον ένα μήνα που είμαστε μαζί κάθε 3-4 χρόνια, μου δίνεις συμπυκνωμένη όλη την αγάπη που δεν μπορείς να μου δώσεις από το τηλέφωνο. Miss you! Στο θείο μου **Γιάννη** ("βλάκας"), στον άνθρωπο που με έβγαλε για το πρώτο μου ποτό, με πήγε σε συναυλίες, που προσπαθησε να με μάθει να οδηγάω μηχανάκι και γενικά προσπάθησε να με κάνει cool από geek. Είσουν ο μεγάλος αδερφός που δεν είχα ποτέ. Πρόσεχε τον ευατό σου γιατί βασίζομαι σε σένα να γεράσουμε μαζί. Στη θεία μου **Δωροθέα**, που με αγάπησε και εμπιστεύτηκε σα δικό της ανήψι. Στα ξαδερφάκια μου **Χρήστο** και **Γιάννη**, που για πάντα θα είναι τα παιδιά που άφησα στην Ελλάδα πριν σχεδόν 8 χρόνια. Κάθε φορά που σας βλέπω έχετε μεγαλώσει τόσο πολύ. Μέσα μου στεναχωριέμαι που δε ήμουν εκεί να σας δω να μεγαλώνετε αλλά ξέρω πως η υπόλοιπη οικογένεια σας προσέχει. Ελπίζω να μη συνεχίσετε να βγάζετε το λάδι των γονιών σας!

And last but definitely not least, **Arancha.** How can words describe what I feel about you? You have been next to me in every single second of this journey. It is simply impossible to express the amount of gratitude I have for you. You shared my joy in moments of success, you picked me up in moments of failure, you guided me through dark paths, you lifted me when things seemed lost and you were always there for me. Your beautiful smile and your super hugs were the things to look for after long hours in the lab. Your love has no limits and I am the luckiest person on earth to have you by my side. You complete my life in ways I could never express. The addition of **Alexaki** to our team has been one of the happiest

moments of my life. It is amazing how someone so small can mean so much for me! I don't know if you will follow your parents' path in science but my only advice to you is: Do whatever makes you happy! Arancha & Alexaki you are my North Star(s).

6 REFERENCES

1. Gosio B. Contributo all'etiologia della pellagra; ricerche chimiche e batteriologische sulle alterazioni del mais. Giornale della Reale Accademia di Medicina di Torino 1893;61:484-7.
2. Ehrlich P, Hata S. Die experimentelle Chemotherapie der Spirillosen (Syphilis, Rückfallfieber, Hühnerspirillose, Frambösie). Berlin,: J. Springer; 1910.
3. Domagk G. Ein Beitrag zur Chemotherapie der bakteriellen Infektionen. Deutsch Med Wochenschrift 1935;61:250-3.
4. Fine MJ, Auble TE, Yealy DM, et al. A prediction rule to identify low-risk patients with community-acquired pneumonia. The New England journal of medicine 1997;336:243-50.
5. WHO Model List of Essential Medicines (20th List). 2017. (Accessed 07 Jun, 2017,
6. Ball P. Quinolone generations: natural history or natural selection? J Antimicrob Chemoth 2000;46:17-24.
7. Pogliano J, Pogliano N, Silverman JA. Daptomycin-mediated reorganization of membrane architecture causes mislocalization of essential cell division proteins. J Bacteriol 2012;194:4494-504.
8. Wilson DN, Schluenzen F, Harms JM, Starosta AL, Connell SR, Fucini P. The oxazolidinone antibiotics perturb the ribosomal peptidyl-transferase center and effect tRNA positioning. Proceedings of the National Academy of Sciences 2008;105:13339-44.
9. Sharma D, Cukras AR, Rogers EJ, Southworth DR, Green R. Mutational Analysis of S12 Protein and Implications for the Accuracy of Decoding by the Ribosome. Journal of Molecular Biology 2007;374:1065-76.
10. Jorgensen JH, Ferraro MJ. Antimicrobial susceptibility testing: a review of general principles and contemporary practices. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 2009;49:1749-55.
11. Ericsson HM, Sherris JC. Antibiotic sensitivity testing. Report of an international collaborative study. Acta Pathol Microbiol Scand B Microbiol Immunol 1971;217:Suppl 217:1+.
12. CLSI. Performance standards for antimicrobial susceptibility testing; 15th informational supplement. CLSI, Wayne, PA 2005.
13. Bauer AW, Kirby WM, Sherris JC, Turck M. Antibiotic susceptibility testing by a standardized single disk method. Am J Clin Pathol 1966;45:493-6.
14. EUCAST. Determination of antimicrobial susceptibility test breakpoints. Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases 2000;6:570-2.
15. Baker CN, Stocker SA, Culver DH, Thornsberry C. Comparison of the E Test to agar dilution, broth microdilution, and agar diffusion susceptibility testing techniques by using a special challenge set of bacteria. Journal of clinical microbiology 1991;29:533-8.
16. Prakash V, Lewis JS, 2nd, Jorgensen JH. Vancomycin MICs for methicillin-resistant Staphylococcus aureus isolates differ based upon the susceptibility test method used. Antimicrob Agents Chemother 2008;52:4528.
17. Richter S FM. Susceptibility Testing Instrumentation and Computerized Expert Systems for Data Analysis and Interpretation. In: Versalovic J CK, Funke G, Jorgensen J, Landry M, Warnock D, ed. Manual of Clinical Microbiology, 10th Edition. Washington, DC: ASM Press; 2011:1144-54.
18. Montgomery K, Raymundo L, Drew WL. Chromogenic cephalosporin spot test to detect beta-lactamase in clinically significant bacteria. Journal of clinical microbiology 1979;9:205-7.

19. Lefevre CK, Singer VL, Kang HC, Haugland RP. Quantitative nonradioactive CAT assays using fluorescent BODIPY 1-deoxychloramphenicol substrates. Biotechniques 1995;19:488-93.
20. Zobell CE. The Effect of Solid Surfaces upon Bacterial Activity. J Bacteriol 1943;46:39-56.
21. Costerton JW, Cheng KJ, Geesey GG, et al. Bacterial biofilms in nature and disease. Annu Rev Microbiol 1987;41:435-64.
22. Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. Science (New York, NY) 1999;284:1318-22.
23. Donlan RM, Costerton JW. Biofilms: survival mechanisms of clinically relevant microorganisms. Clinical microbiology reviews 2002;15:167-93.
24. Kolter R, Losick R. One for all and all for one. Science (New York, NY) 1998;280:226-7.
25. O'Toole GA, Kolter R. Initiation of biofilm formation in Pseudomonas fluorescens WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. Mol Microbiol 1998;28:449-61.
26. Wimpenny J, Manz W, Szewzyk U. Heterogeneity in biofilms. FEMS Microbiol Rev 2000;24:661-71.
27. Wolfaardt GM, Lawrence JR, Robarts RD, Caldwell SJ, Caldwell DE. Multicellular organization in a degradative biofilm community. Appl Environ Microbiol 1994;60:434-46.
28. Molin S, Tolker-Nielsen T. Gene transfer occurs with enhanced efficiency in biofilms and induces enhanced stabilisation of the biofilm structure. Curr Opin Biotechnol 2003;14:255-61.
29. Lapaglia C, Hartzell PL. Stress-Induced Production of Biofilm in the Hyperthermophile Archaeoglobus fulgidus. Appl Environ Microbiol 1997;63:3158-63.
30. Fanning S, Mitchell AP. Fungal biofilms. PLoS pathogens 2012;8:e1002585.
31. Garcia-Meza JV, Barrangue C, Admiraal W. Biofilm formation by algae as a mechanism for surviving on mine tailings. Environ Toxicol Chem 2005;24:573-81.
32. Marsh PD, Bradshaw DJ. Dental plaque as a biofilm. J Ind Microbiol 1995;15:169-75.
33. Drenkard E, Ausubel FM. Pseudomonas biofilm formation and antibiotic resistance are linked to phenotypic variation. Nature 2002;416:740-3.
34. Anderson GG, Palermo JJ, Schilling JD, Roth R, Heuser J, Hultgren SJ. Intracellular bacterial biofilm-like pods in urinary tract infections. Science (New York, NY) 2003;301:105-7.
35. Hall-Stoodley L, Costerton JW, Stoodley P. Bacterial biofilms: from the natural environment to infectious diseases. Nat Rev Microbiol 2004;2:95-108.
36. Vuong C, Voyich JM, Fischer ER, et al. Polysaccharide intercellular adhesin (PIA) protects Staphylococcus epidermidis against major components of the human innate immune system. Cellular microbiology 2004;6:269-75.
37. Flemming HC, Wingender J. The biofilm matrix. Nat Rev Microbiol 2010;8:623-33.
38. Davies DG, Parsek MR, Pearson JP, Iglewski BH, Costerton JW, Greenberg EP. The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science (New York, NY) 1998;280:295-8.
39. Vu B, Chen M, Crawford RJ, Ivanova EP. Bacterial extracellular polysaccharides involved in biofilm formation. Molecules 2009;14:2535-54.
40. 2017. (Accessed 7 July,
41. Branda SS, Vik S, Friedman L, Kolter R. Biofilms: the matrix revisited. Trends Microbiol 2005;13:20-6.

42. Gao B, Zhu X, Xu C, Yue Q, Li W, Wei J. Influence of extracellular polymeric substances on microbial activity and cell hydrophobicity in biofilms. Journal of Chemical Technology & Biotechnology 2008;83:227-32.
43. Hausner M, Wuertz S. High rates of conjugation in bacterial biofilms as determined by quantitative in situ analysis. Appl Environ Microbiol 1999;65:3710-3.
44. Dunne WM. Bacterial adhesion: Seen any good biofilms lately? Clinical microbiology reviews 2002;15:155-+.
45. Carpentier B, Cerf O. Biofilms and their consequences, with particular reference to hygiene in the food industry. J Appl Bacteriol 1993;75:499-511.
46. Kaplan JB. Biofilm dispersal: mechanisms, clinical implications, and potential therapeutic uses. J Dent Res 2010;89:205-18.
47. Karatan E, Watnick P. Signals, regulatory networks, and materials that build and break bacterial biofilms. Microbiol Mol Biol Rev 2009;73:310-47.
48. Percival SL, Suleman L, Vuotto C, Donelli G. Healthcare-associated infections, medical devices and biofilms: risk, tolerance and control. Journal of medical microbiology 2015;64:323-34.
49. Conlon BP. Staphylococcus aureus chronic and relapsing infections: Evidence of a role for persister cells: An investigation of persister cells, their formation and their role in S. aureus disease. Bioessays 2014;36:991-6.
50. Costerton JW, Lewandowski Z, Caldwell DE, Korber DR, Lappin-Scott HM. Microbial biofilms. Annu Rev Microbiol 1995;49:711-45.
51. Bryers JD. Medical biofilms. Biotechnol Bioeng 2008;100:1-18.
52. Paju S, Scannapieco FA. Oral biofilms, periodontitis, and pulmonary infections. Oral Dis 2007;13:508-12.
53. Winkelstroter LK, Teixeira FB, Silva EP, Alves VF, De Martinis EC. Unraveling microbial biofilms of importance for food microbiology. Microbial ecology 2014;68:35-46.
54. Beech IB, Sunner J. Biocorrosion: towards understanding interactions between biofilms and metals. Curr Opin Biotechnol 2004;15:181-6.
55. Singh R, Paul D, Jain RK. Biofilms: implications in bioremediation. Trends in Microbiology 2006;14:389-97.
56. Bigger J. TREATMENT OF STAPHYLOCOCCAL INFECTIONS WITH PENICILLIN BY INTERMITTENT STERILISATION. The Lancet;244:497-500.
57. Lewis K. Persister cells. Annu Rev Microbiol 2010;64:357-72.
58. Moyed HS, Broderick SH. Molecular cloning and expression of hipA, a gene of Escherichia coli K-12 that affects frequency of persistence after inhibition of murein synthesis. J Bacteriol 1986;166:399-403.
59. Balaban NQ, Merrin J, Chait R, Kowalik L, Leibler S. Bacterial persistence as a phenotypic switch. Science (New York, NY) 2004;305:1622-5.
60. Hansen S, Lewis K, Vulic M. Role of global regulators and nucleotide metabolism in antibiotic tolerance in Escherichia coli. Antimicrob Agents Chemother 2008;52:2718-26.
61. Li Y, Zhang Y. PhoU is a persistence switch involved in persister formation and tolerance to multiple antibiotics and stresses in Escherichia coli. Antimicrob Agents Chemother 2007;51:2092-9.
62. Maisonneuve E, Gerdes K. Molecular mechanisms underlying bacterial persisters. Cell 2014;157:539-48.
63. Moyed HS, Bertrand KP. hipA, a newly recognized gene of Escherichia coli K-12 that affects frequency of persistence after inhibition of murein synthesis. J Bacteriol 1983;155:768-75.

64. Fung DK, Chan EW, Chin ML, Chan RC. Delineation of a bacterial starvation stress response network which can mediate antibiotic tolerance development. Antimicrob Agents Chemother 2010;54:1082-93.
65. Baba T, Ara T, Hasegawa M, et al. Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol Syst Biol 2006;2:2006 0008.
66. De Groote VN, Verstraeten N, Fauvart M, et al. Novel persistence genes in Pseudomonas aeruginosa identified by high-throughput screening. FEMS microbiology letters 2009;297:73-9.
67. Gerdes K, Maisonneuve E. Bacterial persistence and toxin-antitoxin loci. Annu Rev Microbiol 2012;66:103-23.
68. Korch SB, Henderson TA, Hill TM. Characterization of the hipA7 allele of Escherichia coli and evidence that high persistence is governed by (p)ppGpp synthesis. Mol Microbiol 2003;50:1199-213.
69. Dorr T, Lewis K, Vulic M. SOS response induces persistence to fluoroquinolones in Escherichia coli. PLoS genetics 2009;5:e1000760.
70. Amato SM, Brynildsen MP. Persister Heterogeneity Arising from a Single Metabolic Stress. Current biology : CB 2015;25:2090-8.
71. Kussell E, Kishony R, Balaban NQ, Leibler S. Bacterial persistence: a model of survival in changing environments. Genetics 2005;169:1807-14.
72. Allison KR, Brynildsen MP, Collins JJ. Metabolite-enabled eradication of bacterial persisters by aminoglycosides. Nature 2011;473:216-20.
73. Lebeaux D, Chauhan A, Letoffe S, et al. pH-mediated potentiation of aminoglycosides kills bacterial persisters and eradicates in vivo biofilms. The Journal of infectious diseases 2014;210:1357-66.
74. Levin BR, Rozen DE. Non-inherited antibiotic resistance. Nat Rev Microbiol 2006;4:556-62.
75. Smith PA, Romesberg FE. Combating bacteria and drug resistance by inhibiting mechanisms of persistence and adaptation. Nat Chem Biol 2007;3:549-56.
76. Mulcahy LR, Burns JL, Lory S, Lewis K. Emergence of Pseudomonas aeruginosa strains producing high levels of persister cells in patients with cystic fibrosis. J Bacteriol 2010;192:6191-9.
77. Lafleur MD, Qi Q, Lewis K. Patients with long-term oral carriage harbor high-persister mutants of Candida albicans. Antimicrob Agents Chemother 2010;54:39-44.
78. Harrison JJ, Turner RJ, Ceri H. Persister cells, the biofilm matrix and tolerance to metal cations in biofilm and planktonic Pseudomonas aeruginosa. Environmental microbiology 2005;7:981-94.
79. Swingle EL. Studies on small colony variants of Staphylococcus aureus. J Bacteriol 1935;29:467-89.
80. Edwards AM. Phenotype switching is a natural consequence of Staphylococcus aureus replication. J Bacteriol 2012;194:5404-12.
81. Proctor RA, von Eiff C, Kahl BC, et al. Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections. Nat Rev Microbiol 2006;4:295-305.
82. Atalla H, Gyles C, Jacob CL, Moisan H, Malouin F, Mallard B. Characterization of a Staphylococcus aureus small colony variant (SCV) associated with persistent bovine mastitis. Foodborne Pathog Dis 2008;5:785-99.
83. Karatzas KA, Zervos A, Tassou CC, Mallidis CG, Humphrey TJ. Piezotolerant small-colony variants with increased thermotolerance, antibiotic susceptibility, and low invasiveness in a clonal Staphylococcus aureus population. Appl Environ Microbiol 2007;73:1873-81.

84. Lannergard J, von Eiff C, Sander G, et al. Identification of the genetic basis for clinical menadione-auxotrophic small-colony variant isolates of Staphylococcus aureus. Antimicrob Agents Chemother 2008;52:4017-22.
85. Schaaff F, Bierbaum G, Baumert N, Bartmann P, Sahl HG. Mutations are involved in emergence of aminoglycoside-induced small colony variants of Staphylococcus aureus. Int J Med Microbiol 2003;293:427-35.
86. Besier S, Ludwig A, Ohlsen K, Brade V, Wichelhaus TA. Molecular analysis of the thymidine-auxotrophic small colony variant phenotype of Staphylococcus aureus. Int J Med Microbiol 2007;297:217-25.
87. Lannergard J, Norstrom T, Hughes D. Genetic determinants of resistance to fusidic acid among clinical bacteremia isolates of Staphylococcus aureus. Antimicrob Agents Chemother 2009;53:2059-65.
88. Gao W, Chua K, Davies JK, et al. Two novel point mutations in clinical Staphylococcus aureus reduce linezolid susceptibility and switch on the stringent response to promote persistent infection. PLoS pathogens 2010;6:e1000944.
89. Duval BD, Mathew A, Satola SW, Shafer WM. Altered growth, pigmentation, and antimicrobial susceptibility properties of Staphylococcus aureus due to loss of the major cold shock gene cspB. Antimicrob Agents Chemother 2010;54:2283-90.
90. Tuchscherr L, Medina E, Hussain M, et al. Staphylococcus aureus phenotype switching: an effective bacterial strategy to escape host immune response and establish a chronic infection. EMBO Mol Med 2011;3:129-41.
91. Tuchscherr L, Heitmann V, Hussain M, et al. Staphylococcus aureus small-colony variants are adapted phenotypes for intracellular persistence. The Journal of infectious diseases 2010;202:1031-40.
92. von Eiff C, Becker K, Metze D, et al. Intracellular persistence of Staphylococcus aureus small-colony variants within keratinocytes: a cause for antibiotic treatment failure in a patient with darier's disease. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 2001;32:1643-7.
93. Kriegeskorte A, Konig S, Sander G, et al. Small colony variants of Staphylococcus aureus reveal distinct protein profiles. Proteomics 2011;11:2476-90.
94. Boles BR, Thoendel M, Singh PK. Self-generated diversity produces "insurance effects" in biofilm communities. Proc Natl Acad Sci U S A 2004;101:16630-5.
95. Vesga O, Groeschel MC, Otten MF, Brar DW, Vann JM, Proctor RA. Staphylococcus aureus small colony variants are induced by the endothelial cell intracellular milieu. The Journal of infectious diseases 1996;173:739-42.
96. Armstrong-Esther CA. Carriage patterns of Staphylococcus aureus in a healthy non-hospital population of adults and children. Annals of human biology 1976;3:221-7.
97. Kluytmans J, van Belkum A, Verbrugh H. Nasal carriage of Staphylococcus aureus: epidemiology, underlying mechanisms, and associated risks. Clinical microbiology reviews 1997;10:505-20.
98. Staphylococcus aureus (ID 154). 2017. 21 Jun 2017,
99. Lowy FD. Staphylococcus aureus infections. The New England journal of medicine 1998;339:520-32.
100. Percival SL, McCarty S, Hunt JA, Woods EJ. The effects of pH on wound healing, biofilms, and antimicrobial efficacy. Wound repair and regeneration : official publication of the Wound Healing Society [and] the European Tissue Repair Society 2014;22:174-86.
101. General information about MRSA in healthcare settings. 2017. (Accessed 07 Jun, 2017,

102. Gjodsbol K, Christensen JJ, Karlsmark T, Jorgensen B, Klein BM, Krogfelt KA. Multiple bacterial species reside in chronic wounds: a longitudinal study. Int Wound J 2006;3:225-31.
103. O'Gara JP, Humphreys H. Staphylococcus epidermidis biofilms: importance and implications. Journal of medical microbiology 2001;50:582-7.
104. Otto M. Staphylococcal biofilms. Current topics in microbiology and immunology 2008;322:207-28.
105. Chang S, Sievert DM, Hageman JC, et al. Infection with Vancomycin-Resistant Staphylococcus aureus Containing the vanA Resistance Gene. New England Journal of Medicine 2003;348:1342-7.
106. Jevons MP, Rolinson GN, Knox R. Celbenin-Resistant Staphylococci. Brit Med J 1961;1:124-&.
107. Hughes GB, Chidi CC, Macon WL. Staphylococci in community-acquired infections: Increased resistance to penicillin. Ann Surg 1976;183:355-7.
108. Saravolatz LD, Markowitz N, Arking L, Pohlod D, Fisher E. Methicillin-resistant Staphylococcus aureus. Epidemiologic observations during a community-acquired outbreak. Ann Intern Med 1982;96:11-6.
109. Adcock PM, Pastor P, Medley F, Patterson JE, Murphy TV. Methicillin-resistant Staphylococcus aureus in two child care centers. The Journal of infectious diseases 1998;178:577-80.
110. Maguire GP, Arthur AD, Boustead PJ, Dwyer B, Currie BJ. Emerging epidemic of community-acquired methicillin-resistant Staphylococcus aureus infection in the Northern Territory. Med J Aust 1996;164:721-3.
111. Wieler LH, Ewers C, Guenther S, Walther B, Lubke-Becker A. Methicillin-resistant staphylococci (MRS) and extended-spectrum beta-lactamases (ESBL)-producing Enterobacteriaceae in companion animals: nosocomial infections as one reason for the rising prevalence of these potential zoonotic pathogens in clinical samples. Int J Med Microbiol 2011;301:635-41.
112. Pinho MG, de Lencastre H, Tomasz A. An acquired and a native penicillin-binding protein cooperate in building the cell wall of drug-resistant staphylococci. Proc Natl Acad Sci U S A 2001;98:10886-91.
113. Hanssen AM, Kjeldsen G, Sollid JU. Local variants of Staphylococcal cassette chromosome mec in sporadic methicillin-resistant Staphylococcus aureus and methicillin-resistant coagulase-negative Staphylococci: evidence of horizontal gene transfer? Antimicrob Agents Chemother 2004;48:285-96.
114. Garcia-Alvarez L, Holden MT, Lindsay H, et al. Meticillin-resistant Staphylococcus aureus with a novel mecA homologue in human and bovine populations in the UK and Denmark: a descriptive study. The Lancet Infectious diseases 2011;11:595-603.
115. Hussain M, Wilcox MH, White PJ. The slime of coagulase-negative staphylococci: biochemistry and relation to adherence. FEMS Microbiol Rev 1993;10:191-207.
116. Mack D, Fischer W, Krokotsch A, et al. The intercellular adhesin involved in biofilm accumulation of Staphylococcus epidermidis is a linear beta-1,6-linked glucosaminoglycan: purification and structural analysis. J Bacteriol 1996;178:175-83.
117. Kırmusaoğlu S. Staphylococcal Biofilms: Pathogenicity, Mechanism and Regulation of Biofilm Formation by Quorum-Sensing System and Antibiotic Resistance Mechanisms of Biofilm-Embedded Microorganisms. In: Dhanasekaran D, Thajuddin N, eds. Microbial Biofilms - Importance and Applications. Rijeka: InTech; 2016:Ch. 10.
118. Speziale P, Pietrocola G, Foster TJ, Geoghegan JA. Protein-based biofilm matrices in Staphylococci. Front Cell Infect Microbiol 2014;4:171.
119. Gotz F. Staphylococcus and biofilms. Mol Microbiol 2002;43:1367-78.

120. Cramton SE, Gerke C, Schnell NF, Nichols WW, Gotz F. The intercellular adhesion (ica) locus is present in Staphylococcus aureus and is required for biofilm formation. Infection and immunity 1999;67:5427-33.
121. O'Gara JP. ica and beyond: biofilm mechanisms and regulation in Staphylococcus epidermidis and Staphylococcus aureus. FEMS microbiology letters 2007;270:179-88.
122. Conlon KM, Humphreys H, O'Gara JP. icaR encodes a transcriptional repressor involved in environmental regulation of ica operon expression and biofilm formation in Staphylococcus epidermidis. J Bacteriol 2002;184:4400-8.
123. Valle J, Toledo-Arana A, Berasain C, et al. SarA and not sigmaB is essential for biofilm development by Staphylococcus aureus. Mol Microbiol 2003;48:1075-87.
124. Ulrich M, Bastian M, Cramton SE, et al. The staphylococcal respiratory response regulator SrrAB induces ica gene transcription and polysaccharide intercellular adhesin expression, protecting Staphylococcus aureus from neutrophil killing under anaerobic growth conditions. Mol Microbiol 2007;65:1276-87.
125. Jefferson KK, Pier DB, Goldmann DA, Pier GB. The teicoplanin-associated locus regulator (TcaR) and the intercellular adhesin locus regulator (IcaR) are transcriptional inhibitors of the ica locus in Staphylococcus aureus. J Bacteriol 2004;186:2449-56.
126. Cue D, Lei MG, Luong TT, et al. Rbf promotes biofilm formation by Staphylococcus aureus via repression of icaR, a negative regulator of icaADBC. J Bacteriol 2009;191:6363-73.
127. Pamp SJ, Frees D, Engelmann S, Hecker M, Ingmer H. Spx is a global effector impacting stress tolerance and biofilm formation in Staphylococcus aureus. J Bacteriol 2006;188:4861-70.
128. Mack D, Siemssen N, Laufs R. Parallel induction by glucose of adherence and a polysaccharide antigen specific for plastic-adherent Staphylococcus epidermidis: evidence for functional relation to intercellular adhesion. Infection and immunity 1992;60:2048-57.
129. Gerke C, Kraft A, Sussmuth R, Schweitzer O, Gotz F. Characterization of the N-acetylglucosaminyltransferase activity involved in the biosynthesis of the Staphylococcus epidermidis polysaccharide intercellular adhesin. J Biol Chem 1998;273:18586-93.
130. Rachid S, Ohlsen K, Witte W, Hacker J, Ziebuhr W. Effect of subinhibitory antibiotic concentrations on polysaccharide intercellular adhesin expression in biofilm-forming Staphylococcus epidermidis. Antimicrob Agents Chemother 2000;44:3357-63.
131. Hjelm E, Lundell-Etherden I. Slime production by Staphylococcus saprophyticus. Infection and immunity 1991;59:445-8.
132. Cramton SE, Ulrich M, Gotz F, Doring G. Anaerobic conditions induce expression of polysaccharide intercellular adhesin in Staphylococcus aureus and Staphylococcus epidermidis. Infection and immunity 2001;69:4079-85.
133. Evans E, Brown MR, Gilbert P. Iron chelator, exopolysaccharide and protease production in Staphylococcus epidermidis: a comparative study of the effects of specific growth rate in biofilm and planktonic culture. Microbiology (Reading, England) 1994;140 (Pt 1):153-7.
134. Rachid S, Ohlsen K, Wallner U, Hacker J, Hecker M, Ziebuhr W. Alternative transcription factor sigma(B) is involved in regulation of biofilm expression in a Staphylococcus aureus mucosal isolate. J Bacteriol 2000;182:6824-6.
135. Knobloch JK, Bartscht K, Sabottke A, Rohde H, Feucht HH, Mack D. Biofilm formation by Staphylococcus epidermidis depends on functional RsbU, an activator of the sigB operon: differential activation mechanisms due to ethanol and salt stress. J Bacteriol 2001;183:2624-33.

136. Toledo-Arana A, Merino N, Vergara-Irigaray M, Debarbouille M, Penades JR, Lasa I. Staphylococcus aureus develops an alternative, ica-independent biofilm in the absence of the arlRS two-component system. J Bacteriol 2005;187:5318-29.
137. Beenken KE, Dunman PM, McAleese F, et al. Global gene expression in Staphylococcus aureus biofilms. J Bacteriol 2004;186:4665-84.
138. Francois P, Tu Quoc PH, Bisognano C, et al. Lack of biofilm contribution to bacterial colonisation in an experimental model of foreign body infection by Staphylococcus aureus and Staphylococcus epidermidis. FEMS Immunol Med Microbiol 2003;35:135-40.
139. Fitzpatrick F, Humphreys H, O'Gara JP. Evidence for icaADBC-independent biofilm development mechanism in methicillin-resistant Staphylococcus aureus clinical isolates. Journal of clinical microbiology 2005;43:1973-6.
140. Hennig S, Nyunt Wai S, Ziebuhr W. Spontaneous switch to PIA-independent biofilm formation in an ica-positive Staphylococcus epidermidis isolate. Int J Med Microbiol 2007;297:117-22.
141. Lasa I, Penades JR. Bap: a family of surface proteins involved in biofilm formation. Res Microbiol 2006;157:99-107.
142. Gross M, Cramton SE, Gotz F, Peschel A. Key role of teichoic acid net charge in Staphylococcus aureus colonization of artificial surfaces. Infection and immunity 2001;69:3423-6.
143. Conlon BP, Geoghegan JA, Waters EM, et al. Role for the A domain of unprocessed accumulation-associated protein (Aap) in the attachment phase of the Staphylococcus epidermidis biofilm phenotype. J Bacteriol 2014;196:4268-75.
144. Houston P, Rowe SE, Pozzi C, Waters EM, O'Gara JP. Essential role for the major autolysin in the fibronectin-binding protein-mediated Staphylococcus aureus biofilm phenotype. Infection and immunity 2011;79:1153-65.
145. Rupp ME, Ulphani JS, Fey PD, Bartscht K, Mack D. Characterization of the importance of polysaccharide intercellular adhesin/hemagglutinin of Staphylococcus epidermidis in the pathogenesis of biomaterial-based infection in a mouse foreign body infection model. Infection and immunity 1999;67:2627-32.
146. Patti JM, Allen BL, McGavin MJ, Hook M. MSCRAMM-mediated adherence of microorganisms to host tissues. Annu Rev Microbiol 1994;48:585-617.
147. Kuroda M, Ito R, Tanaka Y, et al. Staphylococcus aureus surface protein SasG contributes to intercellular autoaggregation of Staphylococcus aureus. Biochemical and biophysical research communications 2008;377:1102-6.
148. Latasa C, Solano C, Penades JR, Lasa I. Biofilm-associated proteins. Comptes rendus biologies 2006;329:849-57.
149. O'Neill E, Pozzi C, Houston P, et al. A novel Staphylococcus aureus biofilm phenotype mediated by the fibronectin-binding proteins, FnBPA and FnBPB. J Bacteriol 2008;190:3835-50.
150. Corrigan RM, Miajlovic H, Foster TJ. Surface proteins that promote adherence of Staphylococcus aureus to human desquamated nasal epithelial cells. BMC Microbiol 2009;9:22.
151. Ryding U, Flock JI, Flock M, Soderquist B, Christensson B. Expression of collagen-binding protein and types 5 and 8 capsular polysaccharide in clinical isolates of Staphylococcus aureus. The Journal of infectious diseases 1997;176:1096-9.
152. Heilmann C, Hussain M, Peters G, Gotz F. Evidence for autolysin-mediated primary attachment of Staphylococcus epidermidis to a polystyrene surface. Mol Microbiol 1997;24:1013-24.
153. Heilmann C, Thumm G, Chhatwal GS, Hartleib J, Uekotter A, Peters G. Identification and characterization of a novel autolysin (Aae) with adhesive properties from Staphylococcus epidermidis. Microbiology (Reading, England) 2003;149:2769-78.

154. Chan WC, Coyle BJ, Williams P. Virulence regulation and quorum sensing in staphylococcal infections: competitive AgrC antagonists as quorum sensing inhibitors. J Med Chem 2004;47:4633-41.
155. Boles BR, Horswill AR. Agr-mediated dispersal of Staphylococcus aureus biofilms. PLoS pathogens 2008;4:e1000052.
156. Beenken KE, Mrak LN, Griffin LM, et al. Epistatic relationships between sarA and agr in Staphylococcus aureus biofilm formation. PLoS One 2010;5:e10790.
157. Kong KF, Vuong C, Otto M. Staphylococcus quorum sensing in biofilm formation and infection. Int J Med Microbiol 2006;296:133-9.
158. Beenken KE, Blevins JS, Smeltzer MS. Mutation of sarA in Staphylococcus aureus limits biofilm formation. Infection and immunity 2003;71:4206-11.
159. Tsang LH, Cassat JE, Shaw LN, Beenken KE, Smeltzer MS. Factors contributing to the biofilm-deficient phenotype of Staphylococcus aureus sarA mutants. PLoS One 2008;3:e3361.
160. Pane-Farre J, Jonas B, Forstner K, Engelmann S, Hecker M. The sigmaB regulon in Staphylococcus aureus and its regulation. Int J Med Microbiol 2006;296:237-58.
161. Jefferson KK, Cramton SE, Gotz F, Pier GB. Identification of a 5-nucleotide sequence that controls expression of the ica locus in Staphylococcus aureus and characterization of the DNA-binding properties of IcaR. Mol Microbiol 2003;48:889-99.
162. Cassat J, Dunman PM, Murphy E, et al. Transcriptional profiling of a Staphylococcus aureus clinical isolate and its isogenic agr and sarA mutants reveals global differences in comparison to the laboratory strain RN6390. Microbiology (Reading, England) 2006;152:3075-90.
163. Nair SP, Bischoff M, Senn MM, Berger-Bachi B. The sigma B regulon influences internalization of Staphylococcus aureus by osteoblasts. Infection and immunity 2003;71:4167-70.
164. Nicholas RO, Li T, McDevitt D, et al. Isolation and characterization of a sigB deletion mutant of Staphylococcus aureus. Infection and immunity 1999;67:3667-9.
165. Kullik II, Giachino P. The alternative sigma factor sigmaB in Staphylococcus aureus: regulation of the sigB operon in response to growth phase and heat shock. Arch Microbiol 1997;167:151-9.
166. Wu Y, Wang J, Xu T, et al. The two-component signal transduction system ArlRS regulates Staphylococcus epidermidis biofilm formation in an ica-dependent manner. PLoS One 2012;7:e40041.
167. Fournier B, Hooper DC. A new two-component regulatory system involved in adhesion, autolysis, and extracellular proteolytic activity of Staphylococcus aureus. J Bacteriol 2000;182:3955-64.
168. Rice KC, Bayles KW. Molecular control of bacterial death and lysis. Microbiol Mol Biol Rev 2008;72:85-109, table of contents.
169. Kelly-Quintos C, Cavacini LA, Posner MR, Goldmann D, Pier GB. Characterization of the opsonic and protective activity against Staphylococcus aureus of fully human monoclonal antibodies specific for the bacterial surface polysaccharide poly-N-acetylglucosamine. Infection and immunity 2006;74:2742-50.
170. Rennermalm A, Li YH, Bohaufs L, et al. Antibodies against a truncated Staphylococcus aureus fibronectin-binding protein protect against dissemination of infection in the rat. Vaccine 2001;19:3376-83.
171. Maira-Litran T, Bentancor LV, Bozkurt-Guzel C, O'Malley JM, Cywes-Bentley C, Pier GB. Synthesis and evaluation of a conjugate vaccine composed of Staphylococcus aureus poly-N-acetyl-glucosamine and clumping factor A. PLoS One 2012;7:e43813.

172. Kaplan JB, Ragunath C, Velliyagounder K, Fine DH, Ramasubbu N. Enzymatic detachment of Staphylococcus epidermidis biofilms. Antimicrob Agents Chemother 2004;48:2633-6.
173. Wu JA, Kusuma C, Mond JJ, Kokai-Kun JF. Lysostaphin disrupts Staphylococcus aureus and Staphylococcus epidermidis biofilms on artificial surfaces. Antimicrob Agents Chemother 2003;47:3407-14.
174. Konturek SJ, Konturek JW, Pawlik T, Brzozowski T. Brain-gut axis and its role in the control of food intake. Journal of physiology and pharmacology : an official journal of the Polish Physiological Society 2004;55:137-54.
175. Ley RE, Lozupone CA, Hamady M, Knight R, Gordon JI. Worlds within worlds: evolution of the vertebrate gut microbiota. Nat Rev Microbiol 2008;6:776-88.
176. Dobson AJ, Chaston JM, Newell PD, et al. Host genetic determinants of microbiota-dependent nutrition revealed by genome-wide analysis of Drosophila melanogaster. Nature communications 2015;6:6312.
177. Goodrich JK, Waters JL, Poole AC, et al. Human genetics shape the gut microbiome. Cell 2014;159:789-99.
178. Tims S, Derom C, Jonkers DM, et al. Microbiota conservation and BMI signatures in adult monozygotic twins. The ISME journal 2013;7:707-17.
179. Kovatcheva-Datchary P, Nilsson A, Akrami R, et al. Dietary Fiber-Induced Improvement in Glucose Metabolism Is Associated with Increased Abundance of Prevotella. Cell metabolism 2015;22:971-82.
180. Cotillard A, Kennedy SP, Kong LC, et al. Dietary intervention impact on gut microbial gene richness. Nature 2013;500:585-8.
181. David LA, Materna AC, Friedman J, et al. Host lifestyle affects human microbiota on daily timescales. Genome biology 2014;15:R89.
182. David LA, Maurice CF, Carmody RN, et al. Diet rapidly and reproducibly alters the human gut microbiome. Nature 2014;505:559-63.
183. Qin J, Li R, Raes J, et al. A human gut microbial gene catalogue established by metagenomic sequencing. Nature 2010;464:59-65.
184. Arrieta MC, Stiemsma LT, Amenyogbe N, Brown EM, Finlay B. The intestinal microbiome in early life: health and disease. Frontiers in immunology 2014;5:427.
185. Voreades N, Kozil A, Weir TL. Diet and the development of the human intestinal microbiome. Front Microbiol 2014;5:494.
186. Gill SR, Pop M, Deboy RT, et al. Metagenomic analysis of the human distal gut microbiome. Science (New York, NY) 2006;312:1355-9.
187. O'Hara AM, Shanahan F. The gut flora as a forgotten organ. EMBO reports 2006;7:688-93.
188. Koleva PT, Kim JS, Scott JA, Kozyrskyj AL. Microbial programming of health and disease starts during fetal life. Birth defects research Part C, Embryo today : reviews 2015;105:265-77.
189. Hofstra JJ, Matamoros S, van de Pol MA, et al. Changes in microbiota during experimental human Rhinovirus infection. BMC infectious diseases 2015;15:336.
190. Dethlefsen L, Relman DA. Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation. Proc Natl Acad Sci U S A 2011;108 Suppl 1:4554-61.
191. Mancabelli L, Milani C, Lugli GA, et al. Meta-analysis of the human gut microbiome from urbanized and pre-agricultural populations. Environmental microbiology 2017;19:1379-90.
192. Falony G, Joossens M, Vieira-Silva S, et al. Population-level analysis of gut microbiome variation. Science (New York, NY) 2016;352:560-4.

193. Relman DA. The human microbiome: ecosystem resilience and health. Nutrition reviews 2012;70 Suppl 1:S2-9.
194. Olszak T, An D, Zeissig S, et al. Microbial exposure during early life has persistent effects on natural killer T cell function. Science (New York, NY) 2012;336:489-93.
195. Round JL, O'Connell RM, Mazmanian SK. Coordination of tolerogenic immune responses by the commensal microbiota. Journal of autoimmunity 2010;34:J220-5.
196. Backhed F, Ding H, Wang T, et al. The gut microbiota as an environmental factor that regulates fat storage. Proc Natl Acad Sci U S A 2004;101:15718-23.
197. Bercik P, Collins SM, Verdu EF. Microbes and the gut-brain axis. Neurogastroenterology and motility : the official journal of the European Gastrointestinal Motility Society 2012;24:405-13.
198. Fung TC, Olson CA, Hsiao EY. Interactions between the microbiota, immune and nervous systems in health and disease. Nature neuroscience 2017;20:145-55.
199. Diaz Heijtz R, Wang S, Anuar F, et al. Normal gut microbiota modulates brain development and behavior. Proc Natl Acad Sci U S A 2011;108:3047-52.
200. Bailey MT, Coe CL. Maternal separation disrupts the integrity of the intestinal microflora in infant rhesus monkeys. Developmental psychobiology 1999;35:146-55.
201. Song H, Yoo Y, Hwang J, Na YC, Kim HS. Faecalibacterium prausnitzii subspecies-level dysbiosis in the human gut microbiome underlying atopic dermatitis. The Journal of allergy and clinical immunology 2016;137:852-60.
202. Hevia A, Milani C, Lopez P, et al. Intestinal dysbiosis associated with systemic lupus erythematosus. mBio 2014;5:e01548-14.
203. Takahashi K, Nishida A, Fujimoto T, et al. Reduced Abundance of Butyrate-Producing Bacteria Species in the Fecal Microbial Community in Crohn's Disease. Digestion 2016;93:59-65.
204. Lepage P, Hasler R, Spehlmann ME, et al. Twin study indicates loss of interaction between microbiota and mucosa of patients with ulcerative colitis. Gastroenterology 2011;141:227-36.
205. Alkanani AK, Hara N, Gottlieb PA, et al. Alterations in Intestinal Microbiota Correlate With Susceptibility to Type 1 Diabetes. Diabetes 2015;64:3510-20.
206. Chen J, Chia N, Kalari KR, et al. Multiple sclerosis patients have a distinct gut microbiota compared to healthy controls. Scientific reports 2016;6:28484.
207. Kumar DK, Choi SH, Washicosky KJ, et al. Amyloid-beta peptide protects against microbial infection in mouse and worm models of Alzheimer's disease. Science translational medicine 2016;8:340ra72.
208. Consortium HMP. A framework for human microbiome research. Nature 2012;486:215-21.
209. Consortium HMP. Structure, function and diversity of the healthy human microbiome. Nature 2012;486:207-14.
210. Ding T, Schloss PD. Dynamics and associations of microbial community types across the human body. Nature 2014;509:357-60.
211. Gibson GR, Hutkins R, Sanders ME, et al. Expert consensus document: The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. Nature reviews Gastroenterology & hepatology 2017;14:491-502.
212. Drekonja D, Reich J, Gezahegn S, et al. Fecal Microbiota Transplantation for Clostridium difficile Infection: A Systematic Review. Ann Intern Med 2015;162:630-8.
213. Vrieze A, Van Nood E, Holleman F, et al. Transfer of intestinal microbiota from lean donors increases insulin sensitivity in individuals with metabolic syndrome. Gastroenterology 2012;143:913-6.e7.

214. Bjarnsholt T, Kirketerp-Moller K, Jensen PO, et al. Why chronic wounds will not heal: a novel hypothesis. Wound repair and regeneration : official publication of the Wound Healing Society [and] the European Tissue Repair Society 2008;16:2-10.
215. Percival SL, Hill KE, Malic S, Thomas DW, Williams DW. Antimicrobial tolerance and the significance of persister cells in recalcitrant chronic wound biofilms. Wound repair and regeneration : official publication of the Wound Healing Society [and] the European Tissue Repair Society 2011;19:1-9.
216. Spoering AL, Lewis K. Biofilms and planktonic cells of Pseudomonas aeruginosa have similar resistance to killing by antimicrobials. J Bacteriol 2001;183:6746-51.
217. Stewart PS, Costerton JW. Antibiotic resistance of bacteria in biofilms. Lancet (London, England) 2001;358:135-8.
218. Walters MC, 3rd, Roe F, Bugnicourt A, Franklin MJ, Stewart PS. Contributions of antibiotic penetration, oxygen limitation, and low metabolic activity to tolerance of Pseudomonas aeruginosa biofilms to ciprofloxacin and tobramycin. Antimicrob Agents Chemother 2003;47:317-23.
219. Jesaitis AJ, Franklin MJ, Berglund D, et al. Compromised host defense on Pseudomonas aeruginosa biofilms: characterization of neutrophil and biofilm interactions. J Immunol 2003;171:4329-39.